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# EFFICIENT METHODS AND APPARATUS FOR HIGH-THROUGHPUT PROCESSING OF GENE SEQUENCE DATA

This application claims benefit of the priority of U.S. Provisional Application

- 5 Serial No. 60/274,686 filed March 8, 2001.

## SEQUENCE LISTING

*AK Sub A*  
10-21-03  
OK to enter 10 This patent hereby incorporates by reference a Sequence Listing on compact disc (CD) in accordance with 37 C.F.R. 1.821-1.825. More particularly, two CDs (one original and one duplicate copy) have been submitted to the U.S.P.T.O., each of which includes the Sequence Listing in a file named "seq\_listing" created on 01/10/2002 and having a size of 284 KB.

## BACKGROUND OF THE INVENTION

15 1. Field of the Invention

The present invention relates generally to the processing of gene sequence data with use of a computer, and more particularly to efficient high-throughput processing of gene sequence data to obtain reliable single nucleotide polymorphism (SNP) data and haplotype data.

20 2. Description of the Related Art

Bioinformatics is a field in which genes are analyzed with the use of software. A gene is an ordered sequence of nucleotides that is located at a particular position on a particular chromosome and encodes a specific functional product. A gene could be

several thousand nucleotide base pairs long and, although 99% of the sequences are identical between people, forces of nature continuously pressure the DNA to change.

From generation to generation, systematic processes tend to create genetic equilibria while genetic sampling or dispersive forces create genetic diversity. Through  
5 these forces, a variant or unusual change can become not so unusual – it will eventually find some equilibrium frequency in that population. This is a function of natural selection pressures, random genetic drift, and other variables. Over the course of time, this process happens many times and primary groups having a certain polymorphism (or "harmless" mutation) can give rise to secondary groups that have this  
10 polymorphism, and tertiary, and so on. Such a polymorphism may be referred to as a single nucleotide polymorphism or "SNP" (pronounced "snip"). Among individuals of different groups, the gene sequence of several thousand nucleotide base pairs long could be different at 5 or 10 positions, not just one.

Founder effects have had a strong influence on our modern day population  
15 structure. Since systematic processes, such as mutation and genetic drift, occur more frequently per generation than dispersive process, such as recombination, the combinations of polymorphisms in the gene sequence are fewer than what one would expect from random distributions of the polymorphic sequence among individuals. That is, gene sequence variants are not random distributions but are rather clustered  
20 into "haplotypes," which are strings of polymorphism that describe a multi-component variant of a given gene.

To illustrate, assume there are 10 positions of variation in a gene that is 2000 nucleotide bases long in a certain limited human population. The nucleotide base identifier letters (e.g., G, C, A, and T) can be read and analyzed, and given a “0” for a normal or common letter at the position and a “1” for an abnormal or uncommon letter.

- 5 If this is done for ten people, for example, the following strings of sequence for the polymorphic positions might be obtained:

10	Person 1:	1000100000
	Person 2:	0000000000
	Person 3:	1000100000
	Person 4:	1111100000
	Person 5:	0000000000
	Person 6:	0000000000
	Person 7:	1000100000
15	Person 8:	1000100000
	Person 9:	0100000001
	Person 10:	1000100100

This list is typical of that which would be found in nature. As shown above, the “1000100000” haplotype is present four times out of ten, the “0000000000” haplotype is present three times out of ten, and the “1000100100” haplotype is present one time out of ten. If this analysis is done for a large enough population, one could define all of the haplotypes in the population. The numbers would be far fewer than that expected from a multinomial probability distribution of allele combinations.

The field of bioinformatics has played an important role in the analysis and understanding of genes. The human genome database, for example, has many files of very long sequences that together constitute (at least a rough draft of) the human genome. This database was constructed from five donors and is rich in a horizontal

sense from base one to base one billion. Unfortunately, however, little can be learned from this data about how people genetically differ from one another. Although some public or private databases contain gene sequence data from many different donors or even contain certain polymorphism data, these polymorphism data are unreliable. Such 5 polymorphism data may identify SNPs that are not even SNPs at all, which may be due to the initial use of unreliable data and/or the lack of proper qualification of such data.

In order to discover new SNPs in genes, one must sequence DNA from hundreds of individuals for each of these genes. Typically, a sequence for a given person is about 500 letters long. By comparing the sequences from many different people, DNA base 10 differences can be noticed in about 0.1% - 1.0% of the positions, and these represent candidate SNPs that can be used in screens whose role is to determine the relationship between traits and gene “flavors” in the population. The technical problem inherent to this process of discovery is that more than 1.0% of the letters are different between 15 people in actual experiments because of sequencing artifacts, unreliable data (caused by limitations in the sequencing chemistry, namely that the quality goes down as the sequence gets longer) or software errors.

For example, if the error rate is 3% and 500 people with 500 bases of sequence each are being screened, there are  $(0.03)(500) = 15$  sites of variation within the sequence. If the average frequency of each variant is 5%, and 500 people are being screened, there 20 are  $(0.05)(0.03)(500)(500) = 375$  sequence discrepancies in the data set which represent letters that are potentially different in one person from other people. Finding the “good ones” or true SNPs in these 375 letters is a daunting task because each of them must be

visually inspected for quality, or subject to software that measures this quality inefficiently.

Furthermore, one must first amplify regions of the human genome from many different people before comparing the sequences to one another. To amplify these 5 regions, a map of a gene is drawn and addresses around the regions of the gene are isolated so that the parts of the gene can be read. These regions of the gene may be referred to as coding sequences and the addresses around these regions may be referred to as primer sequences. More specifically, a primer is a single-stranded oligonucleotide that binds, via complementary pairing, to DNA or RNA single-stranded molecules and 10 serves for the priming of polymerases working on both DNA and RNA.

Conventional primer design programs that identify primer sequences have existed for years, but they are not suitable for efficient high-throughput data processing of genomic (very large) sequence data. Some examples of conventional primer design programs are Lasergene available from DNASTar Inc. and GenoMax available from 15 Informax, Inc. Basically, conventional primer design programs pick the best primer pairs within a given sequence and provide many alternates from which the user selects to accomplish a particular objective.

Efficient high-throughput reliable methods are becoming critical for quickly obtaining and analyzing large amounts of genetic information for the development of 20 new treatments and medicines. However, the conventional primer design programs are not equipped for high-throughput processing. For example, they cannot efficiently handle large sequences of data having multiple regions of interest and require a manual

separation of larger design tasks into their component tasks. Such a manual method would be very time consuming for multiple regions of interest in one large sequence. The output data from these programs are also insufficient, as they bear a loose association to the actual positions provided with the input sequence. Finally, although 5 it is important to obtain a large amount of data for accurate assessment, it is relatively expensive to perform amplification over several runs for a large number of sequences. In other words, one large amplification is less expensive to run than several smaller ones covering the same genetic region. Because there are constraints on the upper size limit, several economic and technical variables should be considered when designing 10 such an experiment.

Accordingly, what are needed are methods and apparatus for use in efficient high-throughput processing of gene sequence data for obtaining reliable high-quality SNP and haplotype data.

15

### SUMMARY OF THE INVENTION

The present invention relates generally to the processing of gene sequence data with a computer, and more particularly to efficient high-throughput processing of gene sequence data for obtaining reliable single nucleotide polymorphism (SNP) data and haplotype data. One novel software-based method involves the use of special primer 20 selection rules which operate on lengthy gene sequences, where each sequence has a plurality of coding regions located therein. Such a sequence may have, for example, 100,000 nucleotide bases and 20 identified coding regions.

The primer selection rules may include a rule specifying that all primer pairs associated with the plurality of coding regions be obtained for a single predetermined annealing temperature. This rule could allow for the subsequent simultaneous amplification of many sequences in a single amplification run at the same annealing  
5 temperature. The rule that provides for this advantageous specification requires that each primer sequence has a length that falls within one or more limited ranges of acceptable lengths, and that each primer has a similar G+C nucleotide base pair content. The primer selection rules may also include a rule specifying that a single primer pair be identified for two or more coding regions if they are sufficiently close together. This  
10 rule also provides for efficiency as the single primer pair may be used for the amplification of two or more coding sequences. Yet even another rule specifies that no primer sequence be selected for that which exists in prestored gene family data. This rule is important since it avoids identifying primer pairs that may amplify sequences other than those desired.

15 The method includes the particular acts of reading gene sequence data corresponding to the gene sequence and coding sequence data corresponding to the plurality of coding sequences within the gene sequence; identifying and storing, by following the special primer selection rules, primer pair data within the gene sequence data for one of the coding sequences; repeating the acts of identifying and storing such  
20 that primer pair data are obtained for each sequence of the plurality of coding sequences; and simultaneously amplifying the plurality of coding sequences in gene

sequences from three or more individuals at the predetermined annealing temperature using the identified pairs of primer sequences.

Reliable single nucleotide polymorphism (SNP) data and haplotype data are subsequently identified with use of these amplified sequences. More particularly, the  
5 method includes the additional steps of sequencing the plurality of amplified coding sequences to produce a plurality of nucleotide base identifier strings (which include, for example, nucleotide base identifiers represented by the letters G, A, T, and C); positionally aligning the plurality of nucleotide base identifier strings to produce a plurality of aligned nucleotide base identifier strings; and performing a comparison  
10 amongst aligned nucleotide base identifiers at each nucleotide base position.

At each nucleotide base position where a difference amongst aligned nucleotide base identifiers exists, the method includes the additional steps of reading nucleotide base quality information (for example, phred values) associated with the aligned nucleotide base identifiers where the difference exists; comparing the nucleotide base  
15 quality information with predetermined qualification data; visually displaying the nucleotide base quality information for acceptance or rejection; and if the nucleotide base quality information meets the predetermined qualification data and is accepted, providing and storing resulting data (SNP identification data) that identifies where the difference amongst the aligned base identifiers exists.

20 After providing and storing all of the resulting data that identifies where the differences exist, the method involves the following additional acts. For each aligned nucleotide base identifier at each nucleotide base position where a difference exists, the

method involves the acts of comparing the nucleotide base identifier with a prestored nucleotide base identifier to identify whether the nucleotide base identifier is a variant; and providing and storing additional resulting data that identifies whether the nucleotide base identifier is a variant. The providing and storing of such additional 5 resulting data may involve providing and storing a binary value of '0' for those nucleotide base identifiers that are identified as variants and a binary value of '1' for those nucleotide base identifiers that are not. The accumulated additional resulting data identifies is haplotype identification data.

Advantageously, the methods described herein allow for high-throughput 10 processing of gene sequence data that is quick, efficient, and provides for reliable output data.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a block diagram of a computer system which embodies the present 15 invention;

FIG. 2 is an illustration of software components which may embody or be used to implement the present invention; and

FIGs. 3A-3C form a flowchart describing a method of efficient high-throughput processing of gene sequence data.

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

FIG. 1 is a block diagram of a computer system 100 which embodies the present invention. Computer system 100 includes a network 102 and computer networks 104 and 106. Network 102 is publicly accessible, and a server 108 and a database 110 which are coupled to network 102 are also publicly accessible. On the other hand, computer networks 104 and 106 are private. Each one of computer networks 104 and 106 include one or more computing devices and databases. For example, computer network 104 includes a computing device 112 and a database 114, and computer network 106 includes a computing device 116 and a database 118. The computing devices may 10 include any suitable computing device, such as a personal computer (PC).

Network 102 may be the Internet, where an Internet Service Provider (ISP) is utilized for access to server 108 and database 110. Database 110 stores public domain gene sequence data. Also, the inventive software is preferably used in connection with and executed on computing device 112 of private network 104. Although a preferred 15 computer system is shown and described in relation to FIG. 1, variations are not only possible, but numerous as one skilled in the art would readily understand. For example, in an alternative embodiment, network 102 may be an Intranet and database 110 a proprietary, private DNA sequence database.

The methods described herein may be embodied and implemented in connection 20 with FIG. 1 using software components 200 shown in FIG. 2. The software may be embedded in or stored on a disk 202 or memory 204, and executable within a computer 206 or a processor 208. Thus, the inventive features may exist in a signal-bearing

medium which embodies a program of machine-readable instructions executable by a processing apparatus which perform the methods.

Such software is preferably used in connection with and executed on computing device 112 of private network 104. Preferably, the system functions within the context 5 of a PC network with a central Sun Enterprise server. The program can be loaded and run on any desktop PC that operates using the Linux or Unix operating system. Other versions could also function in a Windows environment. Alternatively, the software could operate on a publicly accessible server and available for use through a public network such as the Internet.

FIGs. 3A-3C form a flowchart which describes a method for efficient high-throughput processing of gene sequence data. This flowchart can be used in connection with software components 200 of FIG. 2 in the systems described in FIG. 1. Beginning at a start block 302 of FIG. 3A, gene sequence data corresponding to a gene sequence and coding sequence data corresponding to a plurality of coding sequences within the 15 gene sequence are read (step 304). Next, primer pair data within the gene sequence data are identified for one of the coding sequences by following a set of primer selection rules (step 306). The set of primer selection rules includes special rules for efficient, high-throughput processing.

For example, the primer selection rules may include a rule specifying that all 20 primer pair data for the plurality of coding regions be obtained for a single predetermined annealing temperature (e.g., 62° Celsius). This rule allows for the subsequent simultaneous amplification of many sequences in a single amplification run

at the predetermined annealing temperature. This primer selection rule further specifies that each primer sequence have a length that falls within one or more limited ranges of acceptable lengths. The primer selection rules may also include a rule specifying that a single primer pair be identified for two or more coding regions if they  
5 are sufficiently close together, which provides for efficiency as the single primer pair can be used for the amplification of two or more coding sequences. As yet another example, the primer selection rules may include a rule specifying that no primer sequence data be selected for that which exists in prestored gene family data, which is important since the program avoids selecting primer pairs that amplify sequences other  
10 than those intended.

Referring back to FIG. 3A, the primer pair data that were identified in step 306 are stored in association with the coding sequence (step 308), and may be displayed or outputted. If additional coding sequences need to be considered (step 310), the next coding sequence is selected (step 312) and steps 306 and 308 are repeated. Thus, the  
15 acts of identifying and storing are repeated such that primer pair data are obtained for each coding sequence within the gene sequence. Once all of the coding sequences have been considered at step 310, the primer sequences are used in the amplification process.

In particular, the plurality of coding sequences in gene sequences from three or more individuals (typically 100s of individuals) are simultaneously amplified in a gene  
20 amplification machine at the predetermined annealing temperature using the identified pairs of primer sequences (step 314). In the embodiment described, the predetermined annealing temperature is 62° Celsius, but in practice it may be any suitable temperature.

Next, the plurality of amplified coding sequences are sequenced to produce a plurality of nucleotide base identifier strings (step 316). Each nucleotide base identifier string corresponds to a respective sequence of the plurality of amplified coding sequences. In the embodiment described, the nucleotide base identifiers are represented by the letters 5 G, A, T, and C. The partial flowchart of FIG. 3A ends at a connector B 318, which connects with connector B 318 of FIG. 3B.

Single nucleotide polymorphism (SNP) data and haplotype data are subsequently identified with use of these amplified sequences. Beginning at connector B 318 of FIG. 3B, each string of the plurality of nucleotide base identifier strings is 10 positionally aligned with the other to produce a plurality of aligned nucleotide base identifier strings (step 320). This may be performed with use of conventional Clustal functionality, which is described later below. Next, a comparison amongst aligned nucleotide base identifiers is performed at a given nucleotide base position (step 322).

If a difference amongst aligned nucleotide base identifiers exists (step 324), 15 nucleotide base quality information associated with the aligned nucleotide base identifiers where the difference exists is read (step 326). This nucleotide base quality information may be, for example, phred values described later below. The nucleotide base quality information is then compared with predetermined qualification data (step 328). Next, the nucleotide base quality information is visually displayed for acceptance 20 or rejection by the end-user (step 330). This step is important because phred values in themselves are not entirely adequate for determining quality. The reason is that phred uses a relative signal-to-noise ratio, but common sequence artifacts often show as

signals having high ratios. If the nucleotide base quality information meets the predetermined qualification data and is accepted (step 332), resulting data (SNP identification data) that identifies where the difference amongst the aligned base identifiers exists is provided (step 334). This resulting data is stored (step 336).

5        If there are additional nucleotide base positions (step 338), the next nucleotide base position is considered (step 340) and steps 322-338 are repeated. Thus, steps 322-338 continue to execute until all of the differences amongst the aligned nucleotide base identifiers are identified. Step 338 is also executed if no difference exists at step 324, if the nucleotide base quality information is not acceptable at step 332, or if the user rejects  
10      the finding based on its visual appearance. The partial flowchart of FIG. 3B ends at a connector C 342, which connects with connector C 342 in FIG. 3C.

After providing and storing all resulting data that identify where differences amongst the aligned nucleotide base identifiers exist, additional acts are performed starting at connector C 342 of FIG. 3C. At a nucleotide base position where a difference  
15      exists, the nucleotide base identifier is compared with a prestored nucleotide base identifier in order to identify whether it is a variant (step 344). The prestored nucleotide base identifier is known from the stored data in step 336. This data could be stored as variant nucleotide bases or as encoded sites (for example major, minor).

Next, additional resulting data that identifies whether a given nucleotide base  
20      identifier is a variant is provided (step 348). This additional resulting data is stored (step 350) and may be displayed or outputted. Where differences do not exist amongst aligned nucleotide base identifiers, it is assumed that no variants exist. Steps 348-350

may involve providing and storing a binary value of '0' for those nucleotide base identifiers that are identified as variants, and a binary value of '1' for those nucleotide base identifiers that are not. If additional nucleotide base positions need to be considered (step 352), then the next nucleotide base position is selected (step 354) and 5 steps 344-352 are repeated. Step 352 is also executed if no difference is found at step 346. Thus, repeating of the acts occurs so that they are performed for each aligned nucleotide base identifier at each nucleotide base position where a difference exists. The repeating of steps ends when all nucleotide base positions have been considered at step 352. The combined additional resulting data provide haplotype identification data 10 (step 356).

Additional Details Regarding Primer Sequence Selection and Amplification.

Regarding steps 302-314 in FIG. 3A above, which may be referred to as the preamplification process, raw human genome data is used and the method basically draws little maps with the data. Additional details regarding the preamplification 15 process will now be described.

Coding sequences are regions within a gene sequence that encode the protein of a gene. RNA is made from DNA only at these positions. When the RNA is turned into protein, the protein sequence is a translation of the DNA sequence at the coding region. The sequence between coding sequences is called intron, which is a DNA section that 20 divides exons. Exons are the DNA segments that store information about the part of the amino acid sequence of the protein.

The object of the present invention is to survey the coding sequences at each coding region for a given gene in many different people, which is time consuming and expensive using conventional approaches. Therefore, a preamplification strategy is designed so that many sequences can be read in an efficient and inexpensive manner.

- 5    Amplification uses two addresses, one in front of the region of interest and one behind it. These two addresses define sites where short pieces of DNA bind and are extended by an enzyme called *thermus aquaticus* (TAQ) polymerease. Preferably, a high fidelity TAQ variant would be used, such as Pfu polymerase. The two pieces of DNA together with the enzyme result in the amplification or geometric increase in the copy number of
- 10   the sequence between the two addresses. After amplification, the software processes read and compare many sequences to one another to find out where people differ. Without amplification, there is too little DNA to read.

One object of the preamplification process is to appropriately select these addresses, which are the primer sequences, for each one of the coding regions.

15   Ordinarily, this is not a trivial task. For any given coding region, there are typically large numbers of potential primer pair solutions from which to select, and often most of these would result in an inefficient or failed amplification because of non-specificity. The preamplification process described herein works in connection with a plurality of coding regions for many genes and identifies a plurality of primer regions so that

20   amplification can be performed in a specific, cost-effective, and efficient manner.

The software program accepts as input: (1) a genome database sequence file, which may be many hundreds of thousands of letters long and downloaded from the

freely available human genome database (default format for convenience); (2) data (e.g., numbers) that indicate where the coding regions are in the input sequence file. The file containing the coding region data (taken from the annotation of a publicly accessible human genome data file) may be referred to as a “join” file because the data in this file  
5 typically resemble the following:

join(8982..9313, 1..81, 17131..17389, 20010..20169, 21754..22353)/gene="CES1 AC020766"

OR

join(81..140,1149..1320,1827..2092,2402..2548,2648..3089)/gene="example gene AC10003"

10

In the second-listed join file above, the first coding region indicated is the region from 81 to 140; the second coding region indicated is from 1149 to 1320, etc. The object is to select a small region of sequence (e.g., 18-22 letters) in front of and behind each coding region in the input sequence file for each coding region identified in the join file.

15 These small sequences are the primers and, for each identified coding region, the program finds a flanking pair of primer sequences. These primer sequences are then named and presented to the user.

Using the two input files, the software is designed to more particularly perform the following in association with steps 302-314 of FIG. 3A:

- 20 (1) Use the numbers in the input join file to identify the coding regions in the input sequence file;
- (2) Identify or select suitable primer regions around coding regions in the most efficient manner (e.g., sometimes the primers will flank a single coding region, and

sometimes they will flank two or even three coding regions if they are close enough to be amplified efficiently);

(3) Select primer pairs for the same annealing temperature (i.e., the temperature required to get them to do their job during amplification). Thus, if one designs ten 5 primer pairs all with the same annealing temperature, say 62° Celsius, they can all be used in an amplification machine together as each amplification run uses a single fixed temperature;

(4) Avoid ambiguous letters (e.g. the letter “n”) when selecting primer regions;

(5) Design primers using a strategy to reduce the chance that the primer will be 10 within what is called a “repeat” region. This strategy involves recognizing poly-A stretches, ensuring that the least amount of intron sequence possible is present between the two primers (as repeats tend to be removed from exon boundaries by buffer space);

(6) Display to the user all of the statistics surrounding the selections (as examples, how many letters exist between two primers of a pair, the precise numerical 15 position of each of the selected primers, etc.); and

(7) Output the primer sequences in a database compatible format (e.g., tab delimited) for easy ordering from primer synthesis vendors.

Now the following input join file

20 join (81..140)/ gene="example gene AC10009"

and the following input sequence file

25

SEQ ID NO: 1

```

1 GAATTCTTTC CAGAAGGCTT TCCATTTACT TTTCCTAGAT TCATCAGAAG AATCATTATC
61 TACAGCAGCT GTAACTGATT GAAATGTATT TTATGAACAA TAAGACTTGA AAGTTAAAAT
5 121 TGCTCCTTTA TCCATGTACT GAAGAATAAA TATTGTGAAA GCAGTCATAA AAACAGAAGT
181 AATCTTTGG TACCTCTGCA TTAGAACCTCT TTATTAACCA GGTGTATTGC CATTCAACAG
241 TAATATTTTG AAAGGAATCT CTATTTTGA GCAGGTTCA ACTTCTGCTT TTTATTTAA
301 ACAGTAGACT TGAAATATTC AGTAACCATG CTATAAAGAG CTATGCTGTA AGACAGCTT
361 TTCTATTTAT AGAGCATGGT TTTGAAATTA TAACAAAGCA TGGGTTTTAT CCTGAAATCA
10 421 TTCATAAATA ACACGTACCA AAACTTAAT ACGGGCTAGC CAGTGTGAGC CAGTGTGACG

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are considered. For the input sequence file, the number of the first letter of a line is shown at the beginning of each line and there are spaces every ten letters. Typically, there is an annotation before the sequence in the file, such as that shown below, which 15 is ignored by the software:

```

LOCUS      AL355303    157796 bp    DNA          HTG      08-SEP-2000
DEFINITION Homo sapiens chromosome 10 clone RP11-445P17, *** SEQUENCING IN
           PROGRESS ***, 19 unordered pieces.
20 ACCESSION   AL355303
VERSION     AL355303.11 GI:10086110
KEYWORDS    HTG; HTGS_PHASE1; HTGS_DRAFT.
SOURCE      human.

```

25 The input join file identifies the coding region, which is underlined in the sequence below:

SEQ ID NO: 1

```

1 GAATTCTTTC CAGAAGGCTT TCCATTTACT TTTCCTAGAT TCATCAGAAG AATCATTATC
61 TACAGCAGCT GTAACTGATT GAAATGTATT TTATGAACAA TAAGACTTGA AAGTTAAAAT
30 121 TGCTCCTTTA TCCATGTACT GAAGAATAAA TATTGTGAAA GCAGTCATAA AAACAGAAGT
181 AATCTTTGG TACCTCTGCA TTAGAACCTCT TTATTAACCA GGTGTATTGC CATTCAACAG
241 TAATATTTTG AAAGGAATCT CTATTTTGA GCAGGTTCA ACTTCTGCTT TTTATTTAA
301 ACAGTAGACT TGAAATATTC AGTAACCATG CTATAAAGAG CTATGCTGTA AGACAGCTT
361 TTCTATTTAT AGAGCATGGT TTTGAAATTA TAACAAAGCA TGGGTTTTAT CCTGAAATCA
35 421 TTCATAAATA GCACGTACCA AGACTTGAAC ACGGGCTAGC CAGTGTGAGC CAGTGTGACG

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Short sequences (e.g., between 18-22 letters) in front of and behind this coding region are selected based on a set of primer selection rules. The program then names these two primer sequences and presents them to the user at the end of the analysis. This is done seamlessly for multiple coding regions identified in the input join file.

- 5 From the example above, the following primer pair data (in small letters) are selected or designed for the given coding region:

SEQ ID NO: 1

10	1 GAATTCTttc cagaaggctt tccatttacT TTTCCTAGAT TCATCAGAAG AATCATTATC 61 TACAGCAGCT GTAAGTGATT <u>GAAATGTATT</u> TTATGAACAA TAAGACTTGA AAGTTAAAAT 121 <u>TGCTCCTTA</u> TCCATGTACT GAAGAATAAA TATTGTGAAA GCAGTCATAA AAACAGAAAGT 181 AATCTTTGG TACCTCTGCA TTAGAACCTCT TTATTAACCA GGTGTATTGC CATTCAACAG 241 TAATATTTG AAAGGAATCT CTATTTTGA GCAGGTTCA ACTTCTGCTT TTTATTTAA 301 ACAGTAGACT TGAAATATTC AGTAACCATG CTATAAAGAG CTATGCTGTA AGACAGCTT 361 TTCTATTTAT AGAGCATGGT TTTGAAATTA TAACAAAGCA TGGGTTTTAT CCTGAAATCA 421 TTCATAAAATA gcacgtacca agacttgaac ACGGGCTAGC CAGTGTGAGC CAGTGTGACG
15	

- Since there are typically about ten important regions in a given sequence, there are typically about twenty short primer sequences which are produced. Oftentimes, however, a single primer pair that flanks two (or more) coding regions is picked so that 20 the actual total number of identified primer pairs will be less than two times the number of coding regions.

The two sequences are also named according to specific rules. Here, the names for the example as TPMTE2-5 and TPMTE2-3 are given. The two primer sequences are presented to the user in the output form below.

- 25 SEQ ID NOs: 2-3

```
TPMTE2-5 ttccagaaggctttccattac
TPMTE2-3 gttcaagtcttggtaacgtgct
```

Note that the TPMTE2-5 sequence is identical to the first picked sequence whereas the second sequence, TPMTE2-3, is the reverse and compliment of the second picked sequence.

In the preferred embodiment, the following set of primer selection rules are used

5 for selecting primer sequences:

Rule 1: The number of combined "G"s and "C"s should be roughly equal the number of combined "A"s and "T"s.

10 Rule 2: There should be no longer than four consecutive "G"s together (e.g., ...GGGG...), four consecutive "C"s together, four consecutive "A"s together, and four consecutive "T"s together.

15 Rule 3: The length of each primer sequence should fall within the range of 18-22 (inclusive). The length is determined by giving a value of four for each "G", four for each "C", two for an "A", and two for a "T", and then calculating the sum of numbers such that the total sum for any selected sequence must equal 62. Thus, depending on the number of "G"s, "C"s, "T"s and "A"s, the total length of sequence necessary to get a value of 62 will usually fall within the range of 18 to 22 letters (inclusive).

20 Rule 4: The number of letters that fall in between the two selected sequences (herein referred to as a "block") should be equal to some rough integer multiple of 420 letters. For example, the number can be 420, 840, 1280, 1700, or 2120 (2120 is the maximum and 420 is the minimum). The number of letters does not need to be exactly 420, 840, or 1280, etc. however, but can be reasonably close; say plus or minus 50 or even 75. This range also can be chosen arbitrarily at first and then modified later. For example, if plus or minus 50 is chosen, the range should be 370-470, 790-890, or 1230-1330, etc.

25 Rule 5: At least one of the primer sequences must be within 100 letters of the beginning or the end of the coding region.

30 Rule 6: If the size of a block is larger than 1400, a third short sequence should be picked to reside roughly at position "700" in that block. This sequence should have the letters "seq" at the end of its name. For example, in the sequence below, the block is 2290 letters long:

35

SEQ ID NOS: 4-5

```

1 GAATTCTttc cagaaggctt tccatTTacT TTTCTAGAT TCATCAGAAG AATCATTATC
61 TACAGCAGCT GTAACTGATT GAAATGTATT TTATGAACAA TAAGACTTGA AAGTTAAAAT
121 TGCTCCTTTA TCCATGTACT GAAGAATAAA TATTGTGAAA GCAGTCATAA AAACAGAAGT
5 181 AATCTTTGG TACCTCTGCA TTAGAACTCT TTATTAACCA GGTGTATTGC CATTCAACAG
241 TAATATTTG AAAGGAATCT CTATTTTGAA GCAGGTTCA ACTTCTGCTT TTTATTTAA
301 ACAGTAGACT TGAAATATTG AGTAACCATG CTATAAAGAG CTATGCTGTA AGACAGCTT
361 TTCTATTTAT AGAGCATGGT TTTGAAATTAA TAACAAAGCA TGGGTTTAT CCTGAAATCA
421 TGCTCCTTTA TCCATGTACT GAAGAATAAA TATTGTGAAA GCAGTCATAA AAACAGAAGT
10 481 AATCTTTGg tacctctgca tttagaactcT TTATTAACCA GGTGTATTGC CATTCAACAG
541 TAATATTTG AAAGGAATCT CTATTTTGAA GCAGGTTCA ACTTCTGCTT TTTATTTAA
601 ACAGTAGACT TGAAATATTG AGTAACCATG CTATAAAGAG CTATGCTGTA AGACAGCTT
661 TTCTATTTAT AGAGCATGGT TTTGAAATTAA TAACAAAGCA TGGGTTTAT CCTGAAATCA
721 TGctcctttg tccatgtact gaagAATAAA TATTGTGAAA GCAGTCATAA AAACAGAAGT
15 ...1000 bases ...
1781 AATCTTTGG TACCTCTGCA TTAGAACTCT TTATTAACCA GGTGTATTGC CATTCAACAG
1841 TAATATTTG AAAGGAATCT CTATTTTGAA GCAGGTTCA ACTTCTGCTT TTTATTTAA
1901 ACAGTAGACT TGAAATATTG AGTAACCATG CTATAAAGAG CTATGCTGTA AGACAGCTT
1961 TTCTATTTAT AGAGCATGGT TTTGAAATTAA TAACAAAGCA TGGGTTTAT CCTGAAATCA
20 2021 TGCTCCTTTA TCCATGTACT GAAGAATAAA TATTGTGAAA GCAGTCATAA AAACAGAAGT
2081 AATCTTTGG TACCTCTGCA TTAGAACTCT TTATTAACCA GGTGTATTGC CATTCAACAG
2141 TAATATTTG AAAGGAATCT CTATTTTGAA GCAGGTTCA ACTTCTGCTT TTTATTTAA
2201 ACAGTAGACT TGAAATATTG AGTAACCATG CTATAAAGAG CTATGCTGTA AGACAGCTT
2261 TTCATAAAATA gcacgtacca agacttgaac
25

```

At the region around the letter at position "700", one cannot find a third short sequence that meets the criteria of having roughly equal G+C and A+T. A suitable sequence around position "723", however, can be found and is shown in lower case. In this example, three sequences are presented to the user: the first two read exactly as they appear in the lower case letters, and the last one being a reverse and compliment of the sequence at position "2270":

SEQ ID NOS: 6-8

```

35 TPMTE2-5 ttccagaaggcttccattac
TPMTE2-seq ggtacctctgcattagaactc
TPMTE2-3 gttcaagtctggtacgtgct

```

The following is a logic summary for the primer identification rules according to  
40 the preferred embodiment:

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(1) Define the smallest block of sequence that surrounds and completely encompasses the coding region and is either 700 (+/-100) letters long, 1400 (+/- 100) letters long, 2100 (+/-100) letters long, 2800 letters long (+/-200). That is, identify the smallest such block from those having a length =  $n^*(700 +/ - 100)$  for  $n = \{1, 2, 3, 4\}$ .

5 2800 letters long (+/-200). That is, identify the smallest such block from those having a length =  $n^*(700 +/ - 100)$  for  $n = \{1, 2, 3, 4\}$ .

10 (2) Find a sequence at the beginning of this  
block such that:

- (a) the sequence is 18-22 letters long;  
(b) the value of the sum of the letters  
is exactly 62, where a G=4, C=4, A=2 and T=2.

15 Put another way,  $\text{Sum } (T)^2 + \text{Sum } (A)^2 + \text{Sum } (G)^4 + \text{Sum } (C)^4 = 62$ . Using this rule,  $G+C$  will be either 9, 10, or 11 since only with these values is it possible to have a sequence that is 18-22 letters long with the sum of values = 64;

- (d) the last letter should be a "G" or a "C", not an "A" or a "T".

(4) After identifying two or more blocks, if two blocks can be constructed in the input sequence such that the end of one block overlaps with the beginning of another, or such that the end of one is within, say 100 letters of the beginning of another, the two blocks are merged, as long as the new merged block is not greater than 2800 (+/-200). It is preferable to have one large block compared to two or more smaller ones. If the blocks are merged, the first sequence selected for the first block and the last sequence selected for the second block forms the two sequences of the new merged block. The second sequence for the first block and the first sequence of the second block are discarded.

The selected sequences are also named by the software, preferably as follows.

There are three parts to the name. The first is the gene which is the same as the input sequence file name. For example, for the gene "TPMT" all sequences the program finds

for the input sequence file will have "TPMT" in the name. In addition, the first block found includes in its name "E1", the second block found includes in its name "E2", the third "E3", and so on. If two blocks are merged, however, both of these tags will be included in the name of the merged block in order. For example, if "E1" and "E2" blocks  
5 are merged, then the characters "E1E2" will be in the new name for the new merged block. Finally, the first sequence found for a block will have the characters "-5" and the second will have the characters "-3".

Below is a naming example where there are five blocks and two sequences for each block, except where blocks "2" and "3" were merged, and the merged block is 1260  
10 (+/-100) letters long and required a third sequence to be selected:

	TPMTE1-5
	TPMTE1-3
15	TPMTE2E3-5
	TPMTE2E3-3
	TPMTE2E3SEQ
20	TPMTE4-5
	TPMTE4-3
	TPMTE5-5
	TPMTE5-3

25 Another way to describe the naming process is presented. The 5-prime and the 3-prime primer may be presented to the user based on the following logic:

- (1) The name of the gene (which is the sequence file name) and block appears in the name of each primer sequence;
- 30 (2) The gene and block name corresponding to the sequence file is provided in front of the name for a block is provided. If the sequence file is named "AHR", for example, the first block name would include "AHRE1" and the second block name would include "AHRE2";

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(3) The "5" prime or "3" prime designation is also presented in the name of the primer. For example, the primers for the first block of the AHR gene would read:

5

AHRE1-5 - *the first sequence found (sequence whose numerical position is least - e.g. at position 60)*

10 AHRE1-3 - *the second sequence found (sequence whose numerical position is most - e.g. at position 420)*

After naming, the sequence of letters for each primer sequence may be presented as follows:

- 15           1. Present the first sequence (called the "5" primer) as it appears in the sequence, letter for letter but without the blank spaces;
- 20           2. Present the second sequence (called the "3" primer) such that
- 25           a. The sequence is reversed such that the end is now the beginning and the beginning is now the end and then,
- 30           b. "A" is substituted for each "T"  
              c. "T" is substituted for each "A"  
              d. "G" is substituted for each "C"  
              e. "C" is substituted for each "G"  
              (For example: "AATTATGCCT" would become "AGGCATAATT")  
              3. Present any third sequence for a block (if necessary because the block is 1260 +/- 100 letters long) as it appears in the input sequence exactly, letter for letter but without blank spaces.

An example output looks like:

35 SEQ ID NOS: 9-14

40           TYRE15 TTGCATGTTGCAAATGATGTCC  
              TYRE13 CAACCCAGGTATCGTTCAC

TYRE25 CCTCTCAAGCACATTGATCAC  
              TYRE23 TATACTGATCTGAGCTGAGGC

45 and so on, until...

TYRE9-5 TAACATTCACACTAATGGCAGC  
              TYRE9-3 TGCTTCTCCTCTAGAGGCTG

The numerical position of each primer sequence relative to the input sequence is preferably presented as well.

5 The following is an example summary of a join file, a gene sequence file  
(including relevant portions only for brevity), and output data, for the gene "CES1  
AC020766". In the gene sequence file below, the coding regions are highlighted in bold  
print.

```
=====  
10 JOIN FILE FOR GENE "CES1 AC020766"  
  
join(80513..81472,81911..82007,82114..82219,85116..85265,89595..89651)/gene="  
CES1 AC020766"  
=====
```

15 SEQ ID NOs: 15-20

GENE SEQUENCE FILE FOR "CES1 AC020766"

```

20      1 aaccttagcaa acacatgatc ttgttatata tagacatcat tatttgtttc ccctctattc
          61 ttctttcaaa tttctgaatc ataaggattt cctgagccctt ggagatcaag gccagcccttg
         121 gcaacatggc gaaatgccat ctctacaaaaa aaaaaaaaaa aaattatcta ggtgtgttgg
         181 caaggccccaaq tggtcccaagc tactcaqaag qctqaaqtqq qaqqattqct tqagccccaaq

```

25 \*  
\*  
\*

30 28561 agtagagtgcc tggcatactc agtaagacta tattgaataa atgaatgaat aaccccgaaa  
 28621 taaaaatgtt aactataaatg tgttatccta ggtctcaaatt cagaatgatc tgaaaggtag  
 28681 gaaaccccccc tgccactgca gagatctcat cttactttt tgtccttatta taatgggaga  
 28741 ctatggcaag aaatttttga tatctacaga atagatctt atttggacca attttcatct  
 28801 ttgtttgatt caataaacag gctaagttct attcacgaag cctataaaaac tccaaaactc  
 28861 caaatatcca catattccta aatatgtcac ctaactctaa tacatataaca acatgtatgag  
 28921 tacacatcct qtccatttt aqaaacttat qcactcatca ctgtacaccc tggatatctag

40 79801 agttaatgca cacagtttg ctagttttgg cttcaaaaatt aattaaactg tatcaatgt  
79861 ttttgaagtg ttaagtcatt tgtagtgcctt agctccttct atagatgagg caaatataca  
79921 aacagattaa actgactttt acagaataat tattcttta ccttgttac atggaaagga  
79981 atcctccatt ttaggatqca cataaaatgc cagcctatgt tgatgacatt gccttaacac

80041 tttttttta agtaattttc caggtagtt aacctgtaaa agaaacagtg gataaacttg  
 80101 aaaatgctaa tagcaaaaaa cacttcagcc atggcacata caaccagaag ccaatgatat  
 80161 ctttcaacta tagaaattag cggttttc tgtttattcc tgaagcagga ttccatattc  
 80221 aagccagaaa ttgtcattca acagaaaaaa tcaggtcaaa acaatcaatc acataatgt  
 5 80281 gcaagacaaa agtatgtgt tatgtgaaga aaaacaaaaa caacaataaa ccgaacttt  
 80341 attttcttga atataatatt gatggcaaga ttgctaagag gtcatccctg tatttagtt  
 80401 agataaaggc ttccagcata gaacactgtt aagaagtaac tgtcaggagc tatgcagaag  
 80461 tcatgagagg caaataatat aaaaactaga aaagcagggtt ttaattttct atagacttta  
 80521 ttacacatta ttatgttacg agacaatgc agataattct taatttatca aatttgtgag  
 10 80581 cttaaataac aaaaatattt gaccctcacc agaaaaacag ataactctaa atctactctg  
 80641 aaaatcta at caattgcgaa gtattaccta ttggagact atgtattata tcaaagataa  
 80701 agctactatt ctcacagaaac atatgggtc attggcagcc aaccaataat gaagtaaata  
 80761 ttctaatatt tggaaaata ctgagaaaac taataatattt tcctggatatt tattttattct  
 15 80821 tgccttaca aaagacttac acatccaaat gagatttagt tagaatagag gtttttagt  
 80881 cagaaaatgt tcaaagtcca atacagtcat ggctaattcag agactagaga accttataa  
 80941 aggttaatg qcttggaaac ctttggaaac tggcgttct tattttgaaac tagcatgtt  
 81001 taatcaaagg tatggatttta atcaaataatc aattaagaat tactggatg cacactcatg  
 81061 ccaaatgaca actaacatgt tatttcctac tatgtactt ctttggatgg agtcagatgg  
 20 81121 cataaaaaaa tattgttacg tatacaataa attttactt tctgttctg ctctctaaag  
 81181 aaaaatcttta tttttcaca taagaagctc atggatcga atgttattt aaaaaagat  
 81241 aggttaatgta caactgggg aaagacagta ctttcaattt catagggaaat ccatgaaaga  
 81301 attaatcatc ataagagaag aatcattttt ccagtagccc cactaccatg aatgatattt  
 81361 tcatgagcct cggccaccc ttcacatggg tattgagaaac ctatcacagg tttcaaccag  
 25 81421 ccaatttcca ttttccatgg tttttttttt gcatattgtt gaaatttccctc ctaagaaaaag  
 81481 gaaaaacaaa tttttttttt tagtgaaccg tatgattttt ttttccatgg cattaaaaac  
 81541 acttcagaat ctaagtgttta taccatgaaag agtcttttac aaatgtgtga cttttgttca  
 81601 ctttgtccaga actatagaaa aagttagttt ctacagggtt accataatc ccatctgcct  
 81661 gagacagtgt tagtgcataa aataacctt gtcctgttatttactgt atcacatttc  
 30 81721 tatctcaaaa ggtatgttta ctttccatgg tatttactgt tcaccctgt ttttttttgc  
 81781 gtgactaatac ctttccatgg ctttccatgg tatttactgtt atataactaa gtttacatc tattcaact  
 81841 ttcagttgc ctgagtttttccatgg aactgttacc aatgtttttaag ttaccaaat cagaagtact  
 81901 ttttttccatgg ttttttttttccatgg gaaaaagaga gtaactccaa ttataactcgat ctttttttgc  
 81961 atgggttctc gtgggttttccatgg ttttttttttccatgg caacaacccaa acatgaaaaaa  
 35 82021 cagcaatttccatgg ttttttttttccatgg aatgttacc aatgttacc aatttttttccatgg aacatttttccatgg  
 82081 gtggtaaaaa aaaaaaaaaaag aaaagaata ctttccatgg ttttttttttccatgg atgttacc  
 82141 agactcaatg ctttccatgg ttttttttttccatgg gtaactccaa ttataactcgat ctttttttgc  
 82201 ttcttcaccaaa ctttccatgg ttttttttttccatgg ttttttttttccatgg caacaacccaa acatgaaaaaa  
 82261 aactgttacc aatgttacc ttttttttttccatgg ttttttttttccatgg aatgttacc  
 40 82321 aaatgttacc ttttttttttccatgg ttttttttttccatgg ttttttttttccatgg aatgttacc  
 82381 gatgttacc aatgttacc ttttttttttccatgg ttttttttttccatgg aatgttacc  
 82441 ttgttacc ttttttttttccatgg ttttttttttccatgg ttttttttttccatgg aatgttacc  
 82501 aaatgttacc ttttttttttccatgg ttttttttttccatgg ttttttttttccatgg aatgttacc  
 82561 aaaagccccca aacccccccagg gggcccccatttttttttccatgg ttttttttttccatgg aaccccccagg  
 82621 ttttttttttccatgg ttttttttttccatgg ttttttttttccatgg ttttttttttccatgg aaccccccagg  
 45 82681 aaatgttacc ttttttttttccatgg ttttttttttccatgg ttttttttttccatgg aaccccccagg  
 82741 gtttttttttccatgg ttttttttttccatgg ttttttttttccatgg ttttttttttccatgg aaccccccagg  
 82801 aaatgttacc ttttttttttccatgg ttttttttttccatgg ttttttttttccatgg aaccccccagg  
 82861 agctcaaaac aaaactgttacc ttttttttttccatgg ttttttttttccatgg aaccccccagg  
 82921 gggcccccatttttttttccatgg ttttttttttccatgg ttttttttttccatgg aaccccccagg  
 50 82981 tgatttttttccatgg ttttttttttccatgg ttttttttttccatgg ttttttttttccatgg aaccccccagg  
 83041 gaatggatgttacc ttttttttttccatgg ttttttttttccatgg ttttttttttccatgg aaccccccagg  
 83101 ctgttttttccatgg ttttttttttccatgg ttttttttttccatgg ttttttttttccatgg aaccccccagg

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\*

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84301 taacgggcca ttttcatct ttgtgaatat tctggataa tggtatcagc agtgctagat  
 84361 cttaggttcc ccagacgtat aacaaggag tgctttgtt cggcttttg gcaagatgat  
 84421 tgcaaaaaag gtaataaaact ctcaacttta tttttcctt cattttaat gatctaattt  
 84481 acacagtaact caatatttg gaaattctaa tctccccaaac gtgaggaagt ggtaggat  
 84541 tagcaaagca ataagtgtt agcaaattgc taatatagtt caagtgaaga acttcagaat  
 84601 ctgcttgaat tctgttaaat gcagcaacta aataaatgcc acctcaccat tttggatgca  
 84661 gtagtgatta ttcctccaaa gcatccagct aacaatgaa ctttattccc tggccacac  
 84721 agatccagtt tgaatttac agatatctca cttccatgg agaattcaca tcagtagaaa  
 84781 ttatattaag aataccctac agctgaaat acaaagctgc agcttactt agaatgttat  
 84841 ttgcattaaa aatcaattt ttatagctct aagattctag agaagctata ttctatttaa  
 84901 tacacataaa caatacaaaa atgatagtaa aagtttaaaa cttagacatc tgtttttaa  
 84961 ataaattaaa gttttaaaac acgcataaaa attcatcgca ctgaaaaaag gaagcaaaca  
 85021 gctttaaagg agtagttgg taaaaacata taaaaaaacc acgcaagtct ccaaggaaaca  
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 15 85141 tctctgtat tgaacacttc atggctcca tttgcaaaa caatctttg tccttcctca  
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 85261 ccaaacctgaa aaacaaatata acccaagag ttatataattc tctacactcc tgtaaacact  
 85321 taaatacata caatgaactt aagattccta taggaccac cctaacttta aggaacttaa  
 85381 gagtgtaaat gaagaaataa gaaaaacagc taactttat tgagcatttta aataattcca  
 20 85441 ggaaccatac taataattt ctacatattt ttttatttta tcctcacaat gaccctataa  
 85501 agtagatact attattgtcc ctattgtaca gataagaaaag ttgaagcttc aaattataag  
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 85621 cttgaggttt taaccactgc atcaagatgt ttgcagggtt taaagatgtat cagaacatgc  
 85681 tctctgactt cttgtgcat atgaaattct aataacaaa tgtaaggcct ccaccattta  
 25 85741 agtagaaagag ataggatata gggcaattt actaattcat ccatacggtg aatgtttata  
 85801 gagtgtttac gatgtgctag acatggactt taatgtaaa aataaacttta tattctaagg  
 85861 gtggaggaag ataataatgtca tatgaatgaa taaaataat tcagaaata aaagtgcata  
 85921 gaaaaaataa gactggctgt tgggttaaag agacaggaat agggctatt taggtcatca  
 30 85981 ggaagagcca ctctgaaaaa atgagacctg aaaaaagtgaa ggaacaagcc acgagaacat  
 86041 ccggtcagcc acgtggagga tgctgtggc atagtgaatg gccatggcta acctggcag  
 86101 gtgggaatgc agttggggtc aaagaacaga aagagggca gtgtgtctca gggagggcgc  
 86161 tgtacgaaag ggtcgaagat gaggccagaa aggccaagtc acacagaatc tgaggggtga  
 86221 ggtagaggc ttccgagttt attaaaacct gtgcagaacc acggagagc ttaagccagg  
 86281 aatgatctg gttgactca gctttaaaaa gttgctcca attacatgtg aggcacaaag  
 35 86341 aaagcgtga gaaaaatggg aggaggaaga tcagttgtt gcttttagaa cagtctagat  
 86401 aagagatgaa gctggcttga acaaagggtgg tggcacttgc aaaaataaacc aaattcagat  
 86461 atagttttaga ggtaaagctaa tggacttcc tcacagatgtt aatgcgggag atgagggaaa  
 86521 gagaaaaata caggctgtct cctatgtctt tggccagatt aactggtagt agtgagaaga  
 40 86581 ctggagaaca ctaagttgtt gaaaatctcc agatttact ttgcagatgt tggcggcga  
 86641 tgcctgtat cccagctatg tggaggctg aggcaggagg atcgcttggg cccaggaatt  
 86701 tgaggagttt gggattgcag tgatcatgcc actgcactcc agtctggcga acggagcaag

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45

88861 atccagtgac agagttcatg tggatttctt gttaaattct aactgcagag ctctaacttt  
 88921 tccctctaag ctccctgagag gcagattggc agctagttc tcgaagaggt ttctgacagc  
 88981 cctgcattgg gtgatttcat tgaagggtttt attttaattt ctgagtcctc ctccccatt  
 50 89041 ccccccacatt agatttca gccatgggtt gtgggtttaa ggacagggt gtatacgatgc  
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 89161 gaattcacag tggcttttca ccgtgtgtc tggggcaaca tagtaagct ttaatgagcc  
 89221 ttagtttctt tatctaaggg aatatggaaat taatatacaac cttaaagaac tgtttaaaat  
 89281 tctaaataaa tattttata acatatgtca ctgtggc aaaaacaaggc cagtttatct  
 55 89341 tagtctacac ccaatacagg tggaaaatctt aacatatttt tgaagggttg ctctgttgag  
 89401 tttttaacc aagaaaatgtt aaactaatgtt caaaacatca ctttcagaag accaaaaatca  
 89461 aaagttttac tacataaaga aaaaaagcac ctttgactct atttataaaat ctgactttt

89521 aaaatgacca aaggaactat aatgtgaaac ccataaaccc aagcttgttt caaaaatacat  
 89581 taaaaaaaaat acttactcct ccacttgccc catgaaccag aacactctct ccagcttca  
 89641 cacaggca gcaaaggaaa gcataagtta catcaccta tttttgaag ctaattaatc  
 89701 tcgggtgttt tcatcatctt aaggaatttc tacccttagt ctggctaaca cttacacaaa  
 89761 cagcaaatgc aaacctgacat acagccccaa atattcccta agctccacag aataaaacaaa  
 89821 gccttcaatt catttattcc ttgaacaaat atttattggg agtctttatg ttccaggcac  
 89881 tatgctgctg gacactggga tgactatgtg gtgctactc tgagtggcta cagtccttgt  
 89941 gggttgtgaa gtaaaaattgc tgagcctgga ggatctggaa tctctcatc ccataatatcc  
 90001 cccacagaaa gggcctcaaa gcaggttat tata>tagctc agtctttatt ctgtggctca  
 90061 gagtaatgtc caagtaaaca cagtagctat ttttttgc caagaaaga aagaaatttt  
 90121 tcttctccat gtcctgtAAC atcaggttgc accagcctt tacttttca gggaggaatg  
 90181 ctgagtttagc aaaggtcaga gagtaggaaa tgcaataaat tctatcacaa agattcccat  
 90241 gtcatcccc tgaaaatgtcc agattctctg gtgaaatggc attttttt tacttccagt  
 90301 tcacatgact acttttctag tatgtactga aaagaaggga catgcagcaa ggcatgaggg  
 90361 gatgcctcac tattccagat ggacggtgcc aatgtcaaaa gccagcagat gctgtgagat  
 90421 ccagatctga ctctcaggaa ggctcttta cttcctcaaa caatgtgggg tggccacact  
 90481 gcagagacat tatagaacat tatgctccac ctgggaaaga gaacagtaac cagagtctg  
 90541 ctcccagcta tgccccaaca gctgagaagt ggcaacaatg agcaataagt gaagcttct  
 90601 cccacactc tgcttagagc tgaaggact gaggacaata tgtaaaagta aaacataaac  
 90661 ataaggggat aggatgacta gtgttaaact atgggatatg aaatacctcc caaagaattt  
 90721 tttcaaaaat tcttataaga tgcccctcaa acactaaaga cacattctca taaatccctg  
 90781 gggcctgggg tgaggggaga aaaagcaggc aaatccccctc ctgaatccctt gcacagagtc  
 90841 gctgtgacag ttaattttat gtgtcaactt gactgggcca aggaacccaa tatttgttcc  
 90901 aacattactc ttttacagaa acagtgttt tttttttt cgaatgagat taacaatgg  
 90961 atagctggat tttgagtaaa gcagatgacc ctctagaatg tgggtgggcc tcataccatc  
 91021 agttgaaggc ttttgttttcaaaagactgac ctccgatgag caagagtaaa ttcagccagc  
 91081 aaactttcta tggacttaaa ctgcacccctc tccttgc tcccatctgc tggcccaccc  
 91141 caacagattt tagactcacc agtcctccac aatttcatgg gtcaactttaaaaatcaat  
 91201 caatctgtgt ggcgtgtgt gtgtgtgtgt gtgtatgtgt acagagtgac tgattcttaa  
 91261 ggaatttata tagagataaa tgatagatca gatcaaataag aagatcaaataat agatagatga  
 91321 ttgactgata gatagacaga cagacacaca tcccggtt ttttctctg gagaacctg

\*

\*

\*

35

147841 acagacagag atagacagag gcagagtcag ggagaggcag agaaagaaaag agaacaagaa  
 147901 agcttaaaga tagtccaaac gcaaagctgt cttaaaaaaa tgcataactctt attactgca  
 147961 acaaagttt ataatctata cattttatga accactaatc cttatattat tcaagatcac  
 40 148021 aacaggggac tcataattata gagtcaagta aatatcatta ccaacattttt atttaacagt  
 148081 ttgtcctcct taattacatg gagaatgata tagtgcattt ttcatgcctt ttttctctt  
 148141 taacaagcca tatgcaggaa agttccatg ctgcgcacaa ataaaagaaa gtttatatttc  
 148201 attccctaana gaaaactgaa aagc

45 =====

SEQ ID NOs: 21-40

=====

OUTPUT FROM PROGRAM

50 NUMBER OF JOINS 4

1. 80513.... 81472
2. 81911.... 82219

3. 85116.... 85265  
 4. 89595.... 89651

5 JOIN NUMBER ----- 1  
 Length of pair 959  
 Starting position of block 79813  
 Block length (700 + pairlength +800) 2459  
 Block ...  
 10 agtttggctagtttggcttcaaaattaattaaactgtatcaatgtatttgaagtgttaagtcatctgtatgcttt  
 agtccttctatagatgaggcaaatacacaacagatcaaactgactttacagaataattattctttaccttgc  
 tacatggaaaggaaatcccttcatttaggatcacataaaatgccagcctatgttatgacattgccttaacacttt  
 ttttaagtaattttacaggtagttaacctgtaaaagaaacagtggataaacttgaaaatgctaatacgaaaaac  
 acttcagccatggcacatacacaaccagaagccaatgatattcattcaactatagaaaattagcgggtttctgttatt  
 15 cctgaaggcaggattccatattcaagccagaaaattgtcattcaacagaaaaatcaggtaaaaacaatcaatcacata  
 atgttagcaagacaaaagtatgtgcttatgtgaagaaaaacaaaaacaacaaaataaccgaactttatttcttgaat  
 ataataattgtatggcaagattgctaagaggtatccctgtattttagataaaggcttcagcatagaacactgt  
 taagaagtaactgtcaggagctatgcagaagtatgtgagaggcaataataaaaactagaaaagcagggtttatt  
 ttctatagactttattacacattatgttacgagacaaatgcagataattcttaatttatcaaatttgtgagctt  
 20 aattaacaaaatatttgcacccctcaccagaaaaacagataactctaaatctactctgaaaatctaattcaattgcgaat  
 gtattacctatttggagactatgtattatataaagataaagctactattctcacagaaacatatgggtcattggca  
 gccaaccaataatgaagtaatattctaattttggaaaatactgagaaaactaataaaattgtcctggatattt  
 tattctgccttacaaaagacttacacatccaaatgagatttagttagaatagaggttttagttcagaaaatgtt  
 25 caaagtccaaatcacatggctatcagagactagagaaccccttataaagtaagttaggcttggaaacccttggaa  
 actgagcagtcttatttgaacttagcatgtttatcaaaggatgaaattataaagctacttcaattaaatcaattgcgaat  
 gaatgcacactcatgccaatgacaactaacatgttatttctactatgtactcttgcattttgacttgcagatggca  
 taaaaaaaaatattgtctactataaaattttactcttcgtctctgctctaaagaaaaatcttatttttcac  
 ataagaagctcatggaatcgaatgttaattaaagaaaaagataggtaagtacaactggggaaagacagacttca  
 attacataggaaatccatgaaagaattaatcatcataagagaataatcattttccagtagccccactaccatgaat  
 30 gatatttcatgagcctcgccaccccttccatggatattgagaacctatcacaggttcaaccagccaatttcca  
 ttccagcttgaagggctgctgcattgtcttgcattttctcttgcatttttttttttttttttttttttttttttttttt  
 ccgtatgatthaatttcagaagcattaaaaacacttcagaatctaagtgttataccatgaaagagtcttcttacaat  
 gtgtgactttgtcaacttgtccagaactatagaaaaagttagttatctacaggtaaccataatccatctgcctg  
 agacagtgttagtgcataaaatcacctgttgcatt  
 35 tacctggatataaattataactgtcacccttagttgccttctggactaattcttaccaactcccacttagtcatata  
 actaagtttaacatctattcaaacatcccacttgcctgacttagggaaactgtaccaatgtttaagttaccaaaatca  
 gaagtacttctttccatcttgcattgttgcatt  
 cgtgggtttatttcaatagtagtacccatgtccaaacaccaatgtaaaaacagcaattctacagttaaagattact  
 40 gtaaaaatagtgttaattgtggaaaacatataaagtgtaaaaaaaaaaaaaaaagaaaaaggaaatcttactatcactc  
 gtcctccatgtgacagaagactcaagtcttactaagattacattagctaacatttcaataattatcaatttcct  
 ttctcaccaacatactctatataataaaagagaaaatgttagagtaagatagaatgtgaaaaactgtaaaatag□

Actual comp position 80450 sequence tatgcagaagtgtgagaggc  
 Reverse comp position 80450 sequence gcctctcatcacttctgcata  
 45 g c t a toalno totalvalue 8 2 4 7 21 62

Actual comp position 81019 sequence tactggaaatgcacactcatgc  
 Reverse comp position 81019 sequence gcatgagtgtgcattccagta  
 g c t a toalno totalvalue 4 6 5 6 21 62

50

JOIN NUMBER ----- 2  
 Length of pair 308  
 Starting position of block 81211  
 Block length (700 + pairlength +800) 1808

Block ...

tggaaatcgaaatgttaattaaagaaaagatagggtaagtacaactggggaaagacagtacacctataattacataggaa  
atccatgaaagaattaatcatcataagagaagaatcatttccagtagccccactaccatgaatgatatttcatg  
agcctcgccaccccttccaatggatattgagaacctatcacaggttcaaccagccaatttcattccagcttgaa  
ggctgtcatattgtgaattcctctaagaaaaggaaaacaatttttttagtgaaccgtatgattt  
atttcagaagcattaaaacacttcagaatctaagtgttataccatgaagagtcttacaaaatgtgtactttt  
tcaacttgtccagaactatagaaaaagtagttatctacagggttaaccataatccatctgcctgagacagtgttag  
tgtacaaaatacctgttgcattactatgtatcacattctatcataactaaggtatgttacctggatata  
aattatactgtcaccctagttgtccttctggtagactaatccttaccaactccacttagtcataactaagtttac  
atctattcaaacttcagctgccttagttaggcaactgtaccaatgtttaagttacaaaatcagaagacttctt  
ttcctaccttggtagggaaaagagagaactccaattataactcgactcccttgcattgtgtctgtgggttatt  
tcaatagtagtacctctgtccacaacatgaaaaacagcaatttacagttaaagattactgtaaaatagtgt  
taaatttgtgtaaaacattaaagtggaaaaaaaaaaaaagaaaaggaatacttactatcactcgccctccatgt  
acagaagactcaagtcttactaagattcattagctaacattcaataattatcaattccttctcaccac  
tacttctatataataaaagagaaatgttagagtaagatagcaagtgtaaaaactgtaaaatagctactatctgtacaag  
atattatagaaatatgttcaaatgatataatgtctacatctttagactaataatgcaaaaattttaaataatct  
aattatataatcagcatgtattccaaaggtagccaggacatctaaactgtataaaaaattttactaaataacattt  
ctgttagaaataaagtttctggattttcaggtcttagactcaactttagtataaaatacttagctgtaaaattt  
tctatctgtaaaataacttcataaagaaaacaataatcaaaagccccaaacccccagggggctccattttatt  
aataaacaaaagaaaagaaaagatattcattagctgttgcatttttagtgcatttttttttttttttttttttttt  
tgttctaaatggtttatcatctgttttagtgcactaactctttggcttggatgttggacgtggcttacaaa  
aagctacacacatctacattatattcattttatTTTaaacacacacacacaaaatgaatccctgtgcccgggattgcac  
taggtaccaggaaatacaaataacataggagctcaaaacaaaacttagttagaaaaagatggaaatactacagtca  
tagctataaagtatggctaaagtacacacattagcagaaaataatcatagaatacagagaaaaaggttaaggttt  
attgcctgccatggtagataaagtccacagagacga□

get a certain total value of 5 + 4 + 5 = 14.

```

Actual comp position 82362 sequence tgtaattccaagggtaccagcc
Reverse comp position 82362 sequence ggctggtacaccttggaaattaca
g c t a t o a l n o total value 4 6 5 6 21 62

```

JOIN NUMBER ----- 3

Length of pair 149

40 Starting position of block 84416  
Block length (700 + pairlength +800) 1649

### Block ...

tgattgaaaaaaaaaggtaataaaactctcaacttattttcccttcattgtaatctaatttacacagactcaa  
tatggaaaattctaattctccccaaacgtgaggaagtggttgaggattgaaagcaataagtgttagcaaattgc  
taatatagtacaagtgaagaacttcagaatctgcattgttaatgcagcaactaataatgccacctc  
catttggatgcagtagtgattattccctccaaagcatccagctaacaatgaactttattccctggccacacagat  
ccagttgttaattacagatattcacccatggagaattcacatcagtagaaaattatattaagaatacctcaca  
gctgcaaatacaagctgcagttacttagaatgttattgcattaaaaatcaattttatagctctaagattt  
agagaagctatattctatttaatacacataaacaataaaaaatgatagtagaaaagttaaaacttagacatctgtt  
tttaaaaattaaagttttaaaacacgcataaaaattcatcgcaactgaaaaaaggaaagcaaacagctttaaggag  
tagttgttaaaaacatattaaaaaccacgcagaactctccaaggaaacaaagttgactttgtaaaacagtgaaaa  
tttaccttaattttatcaatgtattacttctgtgattgaacacttcatggctccatttgcaaaacaatct  
tttgtccctcctcagtagcaccgcactgccccaaatcttaagccataagctctagcaattggcatgctgtaatcca  
acctgaaaaacaataacccaagagttatattctctacactcctgtaaacacttaaatacatacaatgaactt  
aagattctataggaccacccctaactttaaggaacttaagagtgtaaatgaagaaaataagaaaaacagctacttt  
aatttgagcattaaaatattccaggaaccataactaaataattctacatattgtttattctatcctcacaatgacc  
ctataaagttagataacttattgtcccttattgtacagataagaaagttgaagcttcaaattataagtaattggcca

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agtcatatgcggagatggaaacaggagttagaccagtctgactgcagaactttagtttaccactgcatcaagat  
 gttcagggttaaagatgatcagaacatgcctctgacttcttgcatatgaaattctaaataacaatgtaa  
 ggcctccaccatctaagtagaagagataggatatggcaaattaactaattcatccatatggtaatgttataga  
 gtgttacatgtgctagacatgtacttaatgtaaaataacttatattctaagggtggagaaagataatagtc  
 atatgaatgaaaaataattcagggaaaataaaagtctaagaaaaataaagactggctgtgggttaagagacag  
 gaatagggttatttagtcatcaggaagagccactctgaaaaatgagacctgaaaaaagtgaggaacaagccacg  
 agaacatccggtcagccacgtggaggatgtgt□

5 Actual comp position 85062 sequence gcaagtctccaaggaacaaag  
 10 Reverse comp position 85062 sequence ctttgttccttgagacttgc  
     g c t a toalno totalvalue 5   5 2 9 21 62

15 Actual comp position 85563 sequence gatggaaacaggagttagacc  
 Reverse comp position 85563 sequence ggtctaactcctgtttccatc  
     g c t a toalno totalvalue 7 3 3 8 21 62

20 JOIN NUMBER ----- 4  
 Length of pair 56  
 Starting position of block 88895  
 Block length (700 + pairlength +800) 1556  
 Block ...  
 25 attctaactgcagagctctaactttccctctaagctcctgagaggcagattggcagctagttctcgaaagaggtt  
     ctgacagccctgcattgggtgatttcattgaaggctatttaagttctgagtccctccccatccccacat  
     tagcatttcagccatgggtgtgtttaaggacaggctgtatacgtcactccatggatgtcatcaaagtgcag  
     caggcaagcagcagaaggagatagaaggactaagaattcacagtgtggcttaccgtctgtctggggcaacatag  
     gtaagcttaatgagccttagttcattatctaaggaaatatgaaattatcaacctaagaactgtttaaaat  
 30 tctaaataaatatttataacatatgctacttgaaggcaaaaacaaggccagttatcttagtctacacccaaatc  
     aggtggaaaatctaacatatttgaagggtgcttgttagttattaaccaagaatgctaaactaatgacaaa  
     acatcacccatcagaagaccaaaatcaaaaggtttactacataaagaaaaaaagcaccttgcactctattataaattc  
     tgactttaaaatgacccaaaggaaactataatgtgaaacccatataacccaaagcttgcattcaaaatacattaaaaaa  
     atactactccacttgccttgcaccagaacactctccagcttccatcacaggcactgcaaggaaagcata  
 35 agttacatcaccttattttgaagctaattaatctgggtgtttcatcatcttaagaatttctaccctact  
     ggctaacacttacacaaacagcaaatgcacactgacatcacagcccaaatattccataactccacagaataaaca  
     agcctcaattcatttattccatcacaatatttattggagttcttgcactatgtctggacact  
     ggatgactatgtggctacttgcacttgcactgttt  
     tcttgcattcttgcatt  
 40 ctgtggcttagagtaatgtccaagtaaacacagtagtatttttttttttttttttttttttttttttttttttttt  
     catgtctctgaacatcaggtgcaccgcctgtacttt  
     aggaaatgcaataatctatcacaatggatcccattgtcatccccctgaaatgtccagattctctgtgaaatggca  
     tt  
     ggatgcctcacttccatgtggatggcgtccatgtcaaaagccagcagatgtgagatccagatctgactctc  
 45 aggaaggctcttact□

Actual comp position 89543 sequence gtgaaaccataaaccacgc  
 Reverse comp position 89543 sequence gcttgggttatgggttcac  
     g c t a toalno totalvalue 3 7 2 9 21 62

50 Actual comp position 90103 sequence ctccatgtctctgaacatcag  
 Reverse comp position 90103 sequence ctgatgttcagagacatggag  
     g c t a toalno totalvalue 3 7 6 5 21 62

55 =====

An additional rule relating to gene family members may also be included in the set of primer selection rules. Many genes in the human genome are members of gene families, which means that they closely resemble other genes at other positions in the 5 genome. When primer sequences are selected for a certain gene, one may later find that the selected primers are actually undesirably present in these other family members. The cycle of selecting an appropriate primer sequence for a given gene, that is, identifying a candidate primer sequence, searching the public database to find out whether or not it is specific to that gene, identifying that it is not specific to the gene, 10 reselecting another candidate primer sequence, etc., could go on for several loops before an appropriate primer sequence is identified.

An example command for operating the function for this task is:

```
primer611 sult1a1.txt sult1a1join.txt primerout sult1a2.txt sult1a3.txt
```

15

where the program executable command is primer611, the input sequence file within which to find primers is sult1a1.txt, the input join file that tells the program where the coding (exons) regions is sult1a1join.txt , the output file is primerout, and the other two files, sult1a2.txt and sult1a3.txt, are sequence files of family members. The number of 20 gene family files which may be included can be large.

When the program selects a candidate primer in the sult1a1.txt file, it then reads the sult1a2.txt and sult1a3.txt files to see if it is present. If it is present, it discards it and selects another candidate primer. If it is not present in the files, it selects and stores it

and goes on to find the next primer. The program also looks at the family member files in both forward and reverse directions to be complete and eliminate the user from having to format these files to be in the proper coding orientation.

Thus, the software can select primers that are unique to the gene of interest and  
5 can be relied upon for genes that are members of families. This functionality can be added to the functionality of picking the best primers around the exons of a gene for the primer design process -- select the candidate primer only if it is unique to the target file and not present in the gene family files.

To further illustrate the functionality and output, below is a listing of the  
10 primeronly file and a portion of the primerout file (listing the 1<sup>st</sup> three primer pairs). The command used to generate this output is:

```
primer611 topo2a.txt topo2ajoin.txt primerout topo2b.txt chr18.txt.
```

15 The primerout file is defined in the fourth element of the above command and the primeronly file below is created and named automatically. The primerout file has each of the exon regions defined in the topo2ajoin.txt file printed out with "...." before and after the exon, and documents the steps that the program went through when picking the primers. The primerout file lists candidate primer sequences that otherwise  
20 met the primer selection rules, but was found in one of the gene family files and was therefore rejected (see areas that read "FOUND in"). The output presentation allows a user to go back to a specific region and redesign a primer if the primer selected happens

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to be in a repetitive sequence region not screened out with the gene family files. This may be done, for example, by doing a database search.

SEQ ID NOs: 41-62

5 "PRIMERONLY" FILE  
=====

10 topE1E2-5 actgtggaaacagccagtaga  
topE1E2-3 tcttgataaacctcgctgtgtc

15 topE3E4E5-5  
topE3E4E5-3

20 topE6E7E8-5 atgtgccacccttatccag  
topE6E7E8-3 ttagagatgatgaataaagctcc

25 topE9E10E11-5 cccagcctaacaatccatccatcc  
topE9E10E11-3 ccactacgctcgccaattt

30 topE12E13E14-5 aagagaacagtaactccgc  
topE12E13E14-3 cagcactgattccatgcatac

35 topE15-5 gccagaagtttaggttcaag  
topE15-3 cttaactcagtcggcaagctct

40 topE16-5 gcgtgacacatagcaagtgc  
topE16-3 gccagttttcaatagtagcccc

45 topE17E18E19-5 gagaagaaccttgcacatgg  
topE17E18E19-3 ctccaccattactctcaccaa

50 topE20E21E22-5 tgccctgtataccggatatac  
topE20E21E22-3 ttgacaaaagggtatactgctggaa

55 topE23-5 cttctgtctccacacccctcc  
topE23-3 ggagaggtgagagagagatg

60 topE24-5  
topE24-3

65 topE25E26E27-5 aattgtttctcctactaccctc  
topE25E26E27-3 aacccatctcaaagattttaggc

70 topE28E29-5 aatgcctgtattgaattgcagg  
topE28E29-3 taaaaccaggcttggacttgg

SEQ ID NOS: 63-145

## =====

**"PRIMEROUT" FILE**

## =====

5 Gene Name : top

Sequence File : topo2a.txt  
 Join File : top2ajoin.txt  
 10 Output File : primerout

No of Family sequence files: 2  
 Family Sequence File: topo2b.txt  
 Family Sequence File: chr18.txt  
 15 Number of characters in Sequence file : 22080  
 Number of Lines in Sequence file : 2

JOIN Values .... 29

20 1 1 66 topE1  
 2 290 502 topE2  
 3 1443 1616 topE3  
 4 1806 1907 topE4  
 5 2015 2152 topE5  
 25 6 4630 4768 topE6  
 7 5136 5293 topE7  
 8 5586 5711 topE8  
 9 6318 6428 topE9  
 10 6571 6676 topE10  
 30 11 6767 6876 topE11  
 12 8378 8470 topE12  
 13 8770 8884 topE13  
 14 8988 9109 topE14  
 15 10207 10355 topE15  
 35 16 12180 12411 topE16  
 17 12598 12732 topE17  
 18 12852 13052 topE18  
 19 13194 13389 topE19  
 20 14138 14229 topE20  
 40 21 14332 14496 topE21  
 22 14628 14711 topE22  
 23 16803 16934 topE23  
 24 18702 18854 topE24  
 25 19098 19221 topE25  
 45 26 19328 19371 topE26  
 27 19799 19933 topE27  
 28 21275 21474 topE28  
 29 21792 22080 topE29

50 SORTED JOIN Values ....

1 1 66 topE1  
 2 290 502 topE2  
 3 1443 1616 topE3  
 55 4 1806 1907 topE4  
 5 2015 2152 topE5  
 6 4630 4768 topE6

7	5136	5293	topE7
8	5586	5711	topE8
9	6318	6428	topE9
10	6571	6676	topE10
5	11	6767	topE11
	12	8378	topE12
	13	8770	topE13
	14	8988	topE14
	15	10207	topE15
10	16	12180	topE16
	17	12598	topE17
	18	12852	topE18
	19	13194	topE19
15	20	14138	topE20
	21	14332	topE21
	22	14628	topE22
	23	16803	topE23
	24	18702	topE24
	25	19098	topE25
20	26	19328	topE26
	27	19799	topE27
	28	21275	topE28
	29	21792	topE29

25            COMBINED JOIN Values .....

1	1	502	topE1E2
2	1443	2152	topE3E4E5
30	3	4630	topE6E7E8
	4	6318	topE9E10E11
	5	8378	topE12E13E14
	6	10207	topE15
	7	12180	topE16
35	8	12598	topE17E18E19
	9	14138	topE20E21E22
	10	16803	topE23
	11	18702	topE24
	12	19098	topE25E26E27
40	13	21275	topE28E29

Total no of joins : 13

45            PAIR NO : 1            First      1            Second     502            Name  
 topE1E2  
 PAIR Length .....

50            Block Length ..... : 1301

Block starting position..... : 0

n .....

55 nnnattcagtaccaaatttactgtggaaacagccagtagagaatacaagaaaatgttcaaacaggcaagtaataagtgtcttgatccttaatgataaatggtagtatagccattataatggcattaatgattggtttaatttaacataattataaagctattgaagtatggaaaattataagcatatatattaggttattaggactcataaatttatgttattac

ttcccgaggatgtggacttgcattttcatgtttccattcttacttccatagacatggatggataatatggga  
agagctggatgtggaaactcaagcccttcaatggagaagattatacatgttatcaccccccggatggatccacca  
gtttaaaatgcaaaaggctggacaaagatattgttgcactaatggtcagaagagcatatgatattgtggatccacca  
aaatgtcaaaatgtttcttaatggaaataaaactggccat . . . .

5 gagtattttcctggatgttaaggataataaggatattgtaatcattgtcaagtgc  
ccatatgttttgttggatgtttgatgtttgagacagagtctcacactgttgc  
gcacgatctcgctaccgcacccacccaggttcacgcattctctgc  
attacaggtgcctgccaccacactggtaattttgtatttttagtag  
tggtctcgaaacaccagacacctcatgtatccacccgttgc  
acctggcccaaccatgtatttaccacttcacatgttgc  
aatcagctcatttaaacttaccgaaggatattcttctca  
ttatattttctttcttcttcttgataacctcgctgtc  
cactacagctggacccaggtcaagcgatcatcc  
caccatgccagctaattttttttatagagg  
ctctggatctcaactatgttgc  
10 cccaggtcggtcttgc  
15 ctctggctcaagtgtatccaccacctc□

Did not get PRIMER , what to do , DO NOT HAVE ENOUGH CHARACTERS: 1 TO  
DEAL

	Seq ..	tcttgataacacctcgctgtgtc	FOUND	in	:	chr18.txt	at	8964	position
	Seq ..	ttgataacacctcgctgtgtcac	FOUND	in	:	chr18.txt	at	8966	position
20	Seq ..	gataacacctcgctgtgtcacc	FOUND	in	:	chr18.txt	at	8968	position
	Seq ..	ataacacctcgctgtgtcacc	FOUND	in	:	chr18.txt	at	8969	position
	Seq ..	caggctggagtagactgtatg	FOUND	in	:	chr18.txt	at	8988	position
	Seq ..	aggctggagtagactgtatgc	FOUND	in	:	chr18.txt	at	8989	position
	Seq ..	ctggagtagactgtatgcaatc	FOUND	in	:	chr18.txt	at	8992	position
25	Seq ..	ggagtagactgtatgcaatcac	FOUND	in	:	chr18.txt	at	8994	position
	Seq ..	gagtacagtgtatgcaatcacg	FOUND	in	:	chr18.txt	at	8995	position
	Seq ..	agtacagtgtatgcaatcacgg	FOUND	in	:	chr18.txt	at	8996	position
	Seq ..	cagtgtatgcaatcacggctc	FOUND	in	:	chr18.txt	at	9000	position
	Seq ..	gtgatgcaatcacggctcac	FOUND	in	:	chr18.txt	at	9002	position
30	Seq ..	gcaatcacggctcactacag	FOUND	in	:	chr18.txt	at	9007	position
	Seq ..	caatcacggctcactacagc	FOUND	in	:	chr18.txt	at	9008	position
	Seq ..	aatcacggctcactacagcc	FOUND	in	:	chr18.txt	at	9009	position
	Seq ..	tcaagcgatcatcccacctc	FOUND	in	:	chr18.txt	at	9043	position
	Seq ..	aagcgatcatcccacctcag	FOUND	in	:	chr18.txt	at	9045	position
35	Seq ..	gatcatcccacctcagcttc	FOUND	in	:	chr18.txt	at	9049	position
	Seq ..	tcatcccacctcagcttctg	FOUND	in	:	chr18.txt	at	9051	position
	Seq ..	cacctcagcttctggagtag	FOUND	in	:	chr18.txt	at	9057	position
	Seq ..	acctcagcttctggagtagc	FOUND	in	:	chr18.txt	at	9058	position
	Seq ..	ctcagcttctggagtagctg	FOUND	in	:	chr18.txt	at	9060	position
40	Seq ..	tcagcttctggagtagctgg	FOUND	in	:	chr18.txt	at	9061	position
	Seq ..	cttctggagtagctggaaatg	FOUND	in	:	chr18.txt	at	9065	position
	Seq ..	ttctggagtagctggaaatgc	FOUND	in	:	chr18.txt	at	9066	position
	Seq ..	ggagtagctggaaatgcagg	FOUND	in	:	chr18.txt	at	9070	position
	Seq ..	gagtagctggaaatgcaggc	FOUND	in	:	chr18.txt	at	9071	position
45	Seq ..	gtagctggaaatgcaggcag	FOUND	in	:	chr18.txt	at	9073	position
	Seq ..	tagctggaaatgcaggcagc	FOUND	in	:	chr18.txt	at	9074	position
	Seq ..	qqqatctcaactatqtgccc	FOUND	in	:	chr18.txt	at	9139	position

PRIMER 2 actual : -2130704935 ... tctcaactatgtggccaggc

50 Letters 20 g count 4 t count 6 c count 7 a count 3 total  
62

55 reverse : -2130704935 ... gcctggcaacatagtgaga  
topE1E2-3 gcctggcaacatagtgaga

Number of letters between pairs: -2131274831

	PAIR NO :	2	First	1443	Second	2152	Name
5	topE3E4E5						
	PAIR Length .....			709			
	Block Length .....	:		2208			
10	Block starting position.....	:		743			
	tgcctgccaccacacacctggctaattttgtatTTtagtagagacaggTTcaactatgttgccaggctggctcg aacaccagacacctcatgatccaccgttgcctccaaagtgcTggattacaggcatgagccactgcacctggcc caaccatATgtatTTCTaccacTTcatacatatgttCTgaaaAGagaATggatGCCacATTttaatCAGCT 15 catttAAacttaccgaAGGAatttCTTCAAAGAAACACCTAAAATAATTatTCatGTCCTTTTATTTC CTTTCTTCTTCTTgataacCTCGTGTcaccCAGGCTGGAGTACAGTgatGCAATCACGGCTCactACAG CCTGGACCTCCAGGCTCAAGCGATCATCCACCTCAGCTCTGGAGTAGCTGAAATGCAGGCAGCACCATGC CCAGCTAATTTTTTTCTTTAAATAGAGGTGGGATCTCAGTATGTCCTCAGGTGGTCTGAACCTCTGG 20 CTCAGTgatCCACCCACCTCGGCTGTGTCCTTAATGACCATTCCATTGCTATCAGTGAACATCATTGATT GGTTTGGAAAGTCCTCATAGTCATCATTGAAACCTATTAAATAACTTCTTAATACTGTTACCTTAATTCT GTACAGG .....						
	aaaaggatttcgtagttatgtggacatgtatTTgaaggacaAGTGGATGAAACTGGTAACCTCTGAAAGTAATAC atGAACAAGTAAACACACAGGTGGAAAGTGTGTTAACTATGAGTGAAGGAGCTTCAGCAAATTAGCTTGTCAC 25 AGCATTGCTACATCCAAGGTAATTATTCTTAAATTATTAATCATGATTATCTTACATATATGTTCTTATTG TTTTAAATATAAAAGTGGACTTGAATATTGGCTAGCTTAGTATAAGGAGGTTAAATTAGTTAAATGTTGAT TATTATAATTGAGGAACTGAGTTACAGTTGGTATTTCTTATTAGGGTGGCAGACATGTTGATTATGTA GCTGATCAGATTGTGACTAACTGTTGATTTGTGAGAAGAAGAACAGGGTGGTGTGAGTAAAGCACATCA GGTATGTGCTTGGCAGTTCTTTCTAAAGTCAGGAAGAAGAACAGGCTATAAAAGCATGAGTACATT 30 TTTAGTGGCTTAATATCAACTCTATTGCAAGGTGAAAAATCACATGTGGATTTGTTAAATGCTTAATTGAAAC CAACCTTGTACTCTCAGACAAAAGAACATGACTTACACCAAGAGCTTGGATCAACATGCCAATTGAGTGA AAATTATCAAAGCTT .....						
	GAGTACTTAGAGGAAAATAAGAAACACCTGACTTTATTTCCATTGCACTCTTAGCTCTGAGAAACAATG ATTCTTCTCATAGTGAGCTCTCAAGTCTCCCACATCTGAAAAGGAAGTAAAGGGCTTACTTAACTGATT 35 ACCAAAGACTTAATGACCCTCATATTCTAGTATTCTTAAATTACATTTACCTTACATTAGCTTAGATCATT TAATCTAGCTGTTAAACAAACACCTCACTAAATGCTTAAGACTTGTCTTCACTGCAACACATCCAAAATTGAA GTACCTCCATACTCACTGATTGCCCATAAGCAGCCCCCCTCTCAACAAAAAAACAACCTCTTATCTTAG 40 AAAAGCCCCAACCAACCTCTAGGTTGATAAAACAAGAAAGCTGGAGCCTCCCTTATTCCCTCTCTAATC CGGTCAATAAGAATCATCTTGGATGCTGAGTAGCTTCTCACCATTATCTTTGGTTACTACAATAGTT CTAACCTCATACTGTTAAGTCCCTTGGATGCTTTGAGTGACTTTGTGTTAAACACCCATTCTAC 45 TTCACTCTCATTTGAAATCTTCAATGACTTCCACTCAAGGAAAGTCCAAATTCCATAATTGGCCAACAAGAAGA TCTGCTGTAATCTAATTACACCTACTCTCAACTCATCTCAGTGCAGTTCTGTTGATATTGCTGTTGCTTTA AATTACTGAAAGCACAGTGTCTCCCC Seq .. ccattcccttatGCTTACATAG FOUND in : chr18.txt at 921 position Seq .. gaccatcccttatGCTTAC FOUND in : chr18.txt at 9219 position Seq .. tcaagtgtccaccacCTC FOUND in : chr18.txt at 9182 position Seq .. actcctggcTCAAGTgatC FOUND in : chr18.txt at 9172 position Seq .. tgaactcctggcTCAAGTg FOUND in : chr18.txt at 9169 position Seq .. cttgaactcctggcTCAAG FOUND in : chr18.txt at 9167 position 50 Seq .. aggctggTCTGAACTCCTG FOUND in : topo2b.txt at 36055 position PRIMER 1: 1246 ... tcactatgtgcccaggctg Letters 20 g count 5 t count 6 c count 6 a count 3 total 62 55 topE3E4E5-5 tcactatgtgcccaggctg Seq .. gcctaagacttgctttcagtc FOUND in : chr18.txt at 10319 position Seq .. cctccatactcaactgattgc FOUND in : chr18.txt at 10365 position						

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	Seq ..	ctccatactcaactgatggcc	FOUND	in	:	chr18.txt	at	10366	position
	Seq ..	tccatactcaactgatggcc	FOUND	in	:	chr18.txt	at	10367	position
	Seq ..	cactgatggccatacaaggc	FOUND	in	:	chr18.txt	at	10375	position
	Seq ..	ctgattgcccatacaaggcg	FOUND	in	:	chr18.txt	at	10377	position
5	Seq ..	tgatttgcatacaaggcagc	FOUND	in	:	chr18.txt	at	10378	position
	Seq ..	tttgcatacaaggcagccc	FOUND	in	:	chr18.txt	at	10381	position
	Seq ..	ccaaaccaacaccttagttg	FOUND	in	:	chr18.txt	at	10445	position
	Seq ..	taaacaagaaagctggagcc	FOUND	in	:	chr18.txt	at	10467	position
10	Seq ..	caagaaaagctggagcccttc	FOUND	in	:	chr18.txt	at	10471	position
	Seq ..	aagaaaagctggagcccttc	FOUND	in	:	chr18.txt	at	10472	position
	Seq ..	ctgggagccttccttatttc	FOUND	in	:	chr18.txt	at	10479	position
	Seq ..	tgggagccttccttatttc	FOUND	in	:	chr18.txt	at	10480	position
	Seq ..	gaatcatcttggatgctgc	FOUND	in	:	chr18.txt	at	10525	position
15	Seq ..	atcatcttggatgctgcag	FOUND	in	:	chr18.txt	at	10527	position
	Seq ..	atctcttggatgctgcagttag	FOUND	in	:	chr18.txt	at	10530	position
	Seq ..	ctcttggatgctgcagtagc	FOUND	in	:	chr18.txt	at	10532	position
	Seq ..	ggatgctgcagtagcttc	FOUND	in	:	chr18.txt	at	10537	position
	Seq ..	tgctgcagtagcttcacc	FOUND	in	:	chr18.txt	at	10540	position
20	Seq ..	ctggtaaagtcccttccttg	FOUND	in	:	chr18.txt	at	10605	position
	Seq ..	ttcaatgactccactcaggg	FOUND	in	:	chr18.txt	at	10689	position
	Seq ..	atgacttccactcagggaaag	FOUND	in	:	chr18.txt	at	10693	position
	Seq ..	cttccactcagggaaagtcc	FOUND	in	:	chr18.txt	at	10697	position
	Seq ..	ctcagggaaagtccaaattcc	FOUND	in	:	chr18.txt	at	10703	position
25	Seq ..	tggccaacaagaaagatctgc	FOUND	in	:	chr18.txt	at	10730	position
	Seq ..	gccaaacaagaaagatctgctg	FOUND	in	:	chr18.txt	at	10732	position
	Seq ..	cacctaacttctccaaactcatc	FOUND	in	:	chr18.txt	at	10764	position
	Seq ..	cctacttctccaaactcatctc	FOUND	in	:	chr18.txt	at	10766	position
	Seq ..	cttctccaaactcatctcagtg	FOUND	in	:	chr18.txt	at	10770	position
30	Seq ..	ttctccaaactcatctcagtg	FOUND	in	:	chr18.txt	at	10771	position
	Seq ..	ctccaaactcatctcagtgcc	FOUND	in	:	chr18.txt	at	10773	position
	Seq ..	ccaaactcatctcagtgccag	FOUND	in	:	chr18.txt	at	10775	position

Did not get PRIMER , what to do , DO NOT HAVE ENOUGH CHARACTERS: 2208 TO  
DEAL.

35 [View](#) [Edit](#) [Delete](#)

33

PAIR NO : 3 First 4630 Second 5711 Name  
topE6E7E8

PAIR Length ..... 1081

40

45 gatctcagttcactgcaacccgcgcctcccaggtaaaggcaatttcctgcctcagcctcccaaggcagctaggatta  
cagccatctcaccaccaccatgcctggctaccctttttttttttttttttttttagagcggagttcacttt  
gtcacccaggctggagtgcataatggcgatttggctcgctgcaacctctacacctcctgggttcaagcgatttcctg  
cctcagcctcccgagtagctggattacaggtgcccaccaccacggcagctaattttgtattttagtagagccgg

50 ggttcgcctatgtggccaggccgtctcaactcctgacctcggttgcctccacccatggccctctaaagtgt  
gggattataaggcgtgagccaccgtgcctggctaaattgttttaaccactatatctccaacaagttagtcagtgcta  
gcacaatataattatataatgtaaatattattgaacgaatgaacccaaaaggaggcagtcctcctcagtgggtgataacctg  
acatggaaatgtgcacccttatccagaattattgttctacatcttttaattttgaatcattttatttgt  
attaaggctcattgtattctagatttctgatagatcccttctccctaataatgatccctaataatgaatcttctcg  
tttcagg .....

55 cattggctgttgtttagaaagcataactaaactgggtgaagtttaaggcccaagtccagttaaaacaagaagtgtt  
cagctgtaaaacataatagaatcaaggaaattcccaaactcgatgtgcataatgtgcaggtatataatttaataatg  
tttccaaacttttaagtcttatagttgttattttatttcattaaatggcataaccacggatatttattttcccttgaca

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gaataactatattcaacagaataacttgtaaaaatcgcccgttcctattatggaagathtagtcattccatg  
 ttataaataatattgaggtgattatggagtataaaacaagaatgttattatgtatctattacctaacaata  
 atttgctcattatagtaattgtgtttatcacaaggctataaacagcatgtcaagttatatttgaggtt  
 gaactaaatgtctaataatataatgtatattttatggggccgaaactccactgtacgcattatcctg  
 5 actgaggagattcagccaaaacttggctgtttcagccctgggtggggagagacaaatatggggtttccc  
 tcttagagaaaaactcaatgttcgagaagcttctcataagcaggtagaatataagacgatcttcagaatctaa  
 tctaattataatacaagactttatgcttatttaattccctcattaggcattttaaatatttagacaattt  
 gtgcttatttgagaaatttaggtacattgtacgcatttaacagacccctgtatgttagtaattataagctaata  
 gctcaaaatactggagctcaagaaaatccaagcaacataactgttaaattttttttttcaaatttataaac  
 10 gatgctttttttgttatgtccatttcagatcatggaaaatgtcagatatacatcaagattgtgggtct  
 tcagtacaagaaaaactatgaagatgaagattcatgaagacgcgttatggaaagataatgattgacagatc  
 agt ....  
 cagattgttattaaatttttagattgtcaactaaattaagcatgtcttaatttaatttcattttttgccatg  
 aaaataaattacttaaataggagcttatttcattcatctcaatcaacatctaattcagatatgcttatcatatgt  
 15 tggcaaaatacaggtaagtgagtcggatttgaacagaccccttttgcatttttttttttttttttttttttttt  
 agtaggtcagtcataatatttttatttctaaacaattcttttttttttttttttttttttttttttttttttt  
 aggctggagtgcataatgggtcaatctggctactgcaacccctccgcctcatgggtcaagcgttccctgcctcagc  
 ctcccgagttagctggatttgcaggcggatgccaccacaccaactaattttgtatatttttttttttttttttt  
 accatgttggccaggctggctcgaacgcctgacccctcaggcgatccgcctgcggcctccaaagttctggatt  
 20 acagatgttagtaccacgcggccacttgcataacttttttttttttttttttttttttttttttttttttttt  
 tcccacatcaaaggctgtgatattttatccatcacaactggccctctttctgcacatcgtttctggatt  
 atttatcactcccattgtaaaggtagctatgttt  
 tggatatacatataagccccgtcacatgt□

PRIMER 1: 4479 ... atgtgccaccctctatccag

25 Letters 20 g count 3 t count 5 c count 8 a count 4 total  
 62 topE6E7E8-5 atgtgccaccctctatccag

30 PRIMER 2 actual : 6005 ... gagtgcaatggtgcaatcttg

Letters 21 g count 7 t count 6 c count 3 a count 5 total  
 62

35 reverse : 6005 ... caagattgcaccattgcactc  
 topE6E7E8-3 caagattgcaccattgcactc

Number of letters between pairs: 1526

40

\*  
\*  
\*

45 =====

There are two gene family files in this comparison. The topo2b.txt file is a  
 human genome sequence for a gene called topoisomerase 2b, which is highly related to  
 50 the gene of interest, topoisomerase 2a. In the primerout file, many of the candidate

primers the program selected were present in this family member and were therefore rejected. This demonstrates the utility of the functionality of this program. The second family member sits on chromosome 18 and is a pseudogene (a duplicated region of DNA that does not make a real gene -- a serious nuisance for designing primers that are 5 to amplify a single genetic position). The program has accommodated for this as well; it selected a candidate primer that was found in this file a large number of times.

Without this functionality, primers that would amplify three different regions at the same time would be designed: the topo2a region of interest; the topo2b region related to it; and a nuisance region in chromosome 18. Unfortunately, the resulting data 10 would show numerous discrepancies that are not real polymorphisms. These sequences are actually from different genetic positions that are highly similar to one another but not identical. Thus, most of the "SNPs" found in this manner are not SNPs at all. If one tried to genotype people at a "false SNP," they would get incoherent data as they would be looking at three different positions within the genome at the same 15 time. It is important to produce data for single positions at a time so that the data can be accurately read and interpreted.

Advantageously, the rules that the inventive software uses in the preamplification process are different than those of conventional programs in that they are suitable for use in designing high throughput experiments where many different 20 things can be done simultaneously. It is more efficient to do simultaneous amplifications of four or five regions in 500 people, for example, rather than doing them one by one. This is where the rule regarding the fixed predetermined annealing

temperature (e.g., 62° Celsius) comes into play: since all of the primers selected by the program have the same annealing temperature, the work can be done more efficiently.

Another example is where the software automatically decides if a single primer pair can be utilized for two or more coding regions, which saves additional time and expense.

- 5 Furthermore, the rule regarding gene family data is important for generating reliable output data and for efficiency.

The output of the software is also unique. The numbers included in the output use the numbering pattern that exists in the input sequence file (for example, starting at "10003") rather than starting at "1" like most other programs. This means that a primer 10 at position "11234" can be quickly located, whereas in other programs the number for the primer would be "1231" and one would have to perform the math to figure out its location. This is particularly important for those primers that have to be redesigned manually due to having certain characteristics that can only be determined through a database search.

- 15 Additional Details Regarding The Discovery of Reliable SNP and Haplotype Data. The description that follows provides additional details regarding steps 318-342 of FIG. 3B, which may be referred to as part of the post-amplification process. As described earlier, one important goal of the program is to find reliable discrepancies between individuals at a sequence of a particular genetic locus or location in the 20 genome. To do this, the inventive methods use a direct measure of the nucleotide base quality, or “phred” score, of an observed discrepancy (at steps 326-328 of FIG. 3B).

Actual DNA sequence data files, called chromatograms, are utilized as input, as quality information is an inherent part of such files. As is well-known, a sequence chromatogram looks like a series of colorful peaks and valleys. The color of a peak indicates the DNA base present at that position in the sequence. Peaks in a graph for a  
5 good sequence tend to be higher than for a bad sequence, and overlapping peaks tend to indicate poor reliability. Such information is used to determine whether a discrepancy in a sequence alignment represents a good candidate SNP or not.

The functionality of a conventional phred program is used to call the quality of every letter, and the program aligns the sequences and finds where they are "reliably"  
10 different from one another. By reliable, it is meant that the differences in sequence are differences between letters of good quality. An example of one such program is the phred program available from the University of Washington, which ascribes a numerical value to indicate the quality of each letter of a sequence. The phred functionality makes a separate file with all of these numbers, for each letter.

15 DNA sequences from various individuals are aligned using a conventional sequence alignment algorithm (at step 320), such as that provided using conventional Clustal software functions available by and from the EMBL, Heidelberg Germany, and is a re-write of the popular Clustal V program described by Higgins, Bleasby, and Fuchs  
(1991) CABIOS, 8, 189-191 (Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994)  
20 (CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Research, 22:4673-4680). Thus, the sequence alignment file is the first

input file to the program. Any discrepancy that occurs within a neighborhood of other discrepancies is recognized so that the quality value information can be checked. If this information is greater than predetermined quality information, such as a user-defined input value, it is accepted and presented to the user for final acceptance. If not, it is  
5 discarded. The quality control file created from the phred functionality serves as the second input file.

In the sequence within which the discrepancy occurs, positions of the minor letters of the discrepancy are presented to the end-user. This lets the end-user contemporaneously call up the raw DNA sequence chromatogram and find the actual  
10 trace data peak for the letter. This is advantageous because a visual inspection of raw DNA sequence data is the most reliable method of determining whether a discrepancy is valid. While the purpose of the software is to eliminate many time consuming steps, in some cases, borderline quality values nonetheless necessitate its execution. The presentation of the precise position and relevant file names for a discrepancy makes this  
15 step easy to execute. Also, the end-user is shown presentations of discrepancies that do not meet the quality control criteria. This is important because, in some cases, a borderline quality value may conceal good data due to other problems with sequence compressions or peak spacing.

Another important attribute is afforded the software because it can recognize  
20 reliable base deletion polymorphisms. This is performed by parsing the phred quality data for the bases surrounding the deletion in randomly selected sequences which contain the deletion. With conventional programs, if a discrepancy is a deleted base

there is no quality control information to check since no data is produced for a non-base (and there is consequently no phred value for the deleted base). This eliminates any discovery of single base deletion polymorphisms. Deletion polymorphisms are common and, since the goal is to thoroughly document the various genetic haplotypes  
5 in a population, a SNP-finding program that can recognize deletion polymorphisms offers competitive advantages. Not knowing all of the variants in a gene sequence causes the resolution of haplotype-based studies to be sub-optimal, compared to being able to recognize all variants (including deletion polymorphisms).

The software may also incorporate rules to maximize efficiency during these  
10 steps. For example, the program may focus on determining the phred value for discrepancies that fall within a block of sequence with an acceptable average phred value. As another example, the user-defined phred value could be different for different regions of the sequence. In another variation, the program is configured to recognize amino acid differences by translating the sequences and instructed to only  
15 present candidate polymorphisms that result in a change in amino acid sequence.

Example Walk-Through. Input = (1) Clustal W alignment file and (2) phred quality file. The user inputs a minor letter phred quality control value for the current run, as well as a local phred quality control value. For example, the user may enter the values "24" and "17" for the minor letter and local phred quality control values,  
20 respectively. Then, from the first input file, each column (position or slice) of the alignment is analyzed to determine whether the column is homogeneous (i.e., whether each sequence has the same letter at that position) or heterogeneous (i.e. whether there

are two or more different letters at that position).

As an example, consider the following:

SEQ ID NOS: 146-152

	AHRE11-3	AGGGGGTAGATTTAAAAAT-CATGTTAATGTTATTTACT-
5	AHRE11-3-E10	AGGGGGTAGATTTAAAAAT-CATGTTAATGTTATTTACT-
	AHRE11-3a	AGGTGTAAGATTTAAAAATACATGTTAATGTTATTTACT-
	AHRE11-3u	AGGGGTA-GATTCAAAAATACATGTTAATGTTATTTACT-
	14	AGGGGTA-GATTCAAAAATACATGTTAATGTTATTTACT-
10	AHRE11-3-C4	AGGGGTAAGATTTAAAAATACATGTTAATGTTATTTACT-
	AHRE11-3-D5	AGGGGTAAGATTTAAAAATACATGTTAATGTTATTTACT-

The first column of letters is homogeneous. So is the second and third. The fourth is heterogeneous, as is the sixth, etc.

The second input file is the phred quality file, which takes the format of the 1XN matrix below for each sequence. The entry for the first sequence above (AHRE11-3) appears below:

```
>AHRE11-3 folder=AHRE11-3 length=414
8 9 23 24 32 34 27 27 34 34 32 32 34 34 32 32 29 29 26 26 26 26 28 34 31 29 29
20 32 35 35 35 45 45 45 40 35 35 39 32 33 32
```

In this file, the first two letters are of very low quality or reliability because, for biochemical reasons, sequencing reactions routinely have trouble at the beginning of a sequence read.

For each column of the alignment, the software recognize whether there is a discrepancy (i.e., major and minor letters.) If a discrepancy exists, then the following logic is executed:

For each minor letter, read the phred value.  
 For example, in column 14 above, sequence AHRE11-3u has a C but the others have a T. The “C” is a minor letter and it has the value 34.

- Calculate the average phred value for the major letter (G in column 14 above)
- 5 Calculate the average phred value for each minor letter (in column 14 above, there is only one minor so this is the same as the phred value for that letter.)
- Determine the number of major letters.
- Determine the number of minor letters.
- 10 Calculate the average phred value for the block of letters 7 in front and 7 behind the column using all of the input sequences and their quality values. This will be called the local phred quality value.

To process the job, the phred value of the minor letter and average phred value of the  
15 major letter are utilized such that

- If the phred value of any minor letter in the column is greater than the user-defined threshold value,
- 20 And
- If the average phred value of the major letter for the column is above a different threshold value defined by the user,
- Then label the column as accepted and present to the user for visual inspection.

25 Alternatively, a more sophisticated method for determining the worth of a positional column is to use a function to calculate the probability that a column contains a reliable polymorphism using the average quality value for the column, the quality values for the minor letters, the quality value for the region around the column (using  
30 all the sequences), or other variables. For this approach the following logic is utilized:

- 1) A column with a high average major letter phred score and a high minor letter phred score is a better column than one with
- 35 a) a low average major letter phred score and a high minor letter phred score;
- b) a high average major letter phred score and a low minor letter phred score;
- c) a low average major letter phred score and a low minor letter phred score; and

2) A column with a discrepancy in a region of sequence that has a high local phred quality value is better than one in a region with a low local phred quality value.

5

Preferably, a probability function is employed for this task, including variables for that which is measured above. For example, one might use Bayes' theorem to calculate this probability; for every column a vector is created from the variables calculated above and the linear equation:

10

$y = A_1X_1 + A_2X_2 + A_3X_3 \dots A_nX_n$   
giving the vector  $Y = (A_1, A_2, A_3 \dots A_n)$ , where  $A_n$  are parameters.

Then determine a Bayesian estimate  
 15  $p(w|x) = [p(x|w)p(w)] / p(x)$ ,  
 where  $p(w|x)$  = classification score of the column as good or bad or somewhere in between (called the posterior probability),  $p(x)$  is the frequency or uniqueness or worth of this vector, and  $p(w)$  is the frequency or uniqueness of the class.  $P(x|w)$  is the conditional probability that  $x$  is observed given that  $w$  is also observed - in this frequency that vectors of the above  $A_n$  are observed for true SNP columns (determined using other suitable biochemical techniques).

20

25

Once the alignment file has been inspected for every column, the results are presented to the user. For example, if the probability is high that a column contains a reliable polymorphism, then the column is presented to the user along with 7 letters in front and 7 letters behind for each sequence in the alignment. For example,

30 SEQ ID NOS: 153-155

Sequence 1 TTTATCTGACTGGAG  
 Sequence 2 TTTATCTGACTGGAG  
 35 Sequence 3 TTTATCTCACTGGAG

Also, the "average" sequence 200 letters in front and 200 letters behind the column is presented. For example,

SEQ ID NO: 156

	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG
5	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG
	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG
	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG
	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG G/C
	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG
10	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG
	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG
	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG

In the above example, there is only one column with discrepancies; each of the other columns are homogeneous. In practice, this will be unusual and the presentation will

15 look more like the following (note the letters R, Y, M):

SEQ ID NO: 157

	YTTATGCTCG	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG
	ATTATGCTCG	ATTATGCTCG	RTTATGCTCG	ATTATGCTCG	ATTATGCTCG
	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG
20	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG S
	ATTATGCTCG	ATMATGCTCG	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG
	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG
	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG
	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG	ATTATGCTCG

25 Where

	R=A or G
	Y=C or T
	K=G or T
30	M=A or C
	S=G or C
	W=A or T
	N=any base
	B=C, G, or T
35	D=A, G or T
	H=A, C or T
	V=A, C or G

Other information may also be presented, such as the following: (a) for each sequence with a minor letter, the sequence name and the associated phred value for the minor letter; and (b) the local region phred score.

**Example Output.** Below is a file that shows what the software produces as it

5 inspects a single discrepancy.

SEQ ID NOs: 158-220

=====

```

10      k = 70
10      Position of Reference sequence without dashes :
10      65
10      Position of complement sequence: 209

15      Indicator          ^
15

20      QUALITY INFORMATION
20      Discrepancies at position
20      70

20      Minor letter 1:::-::1
20      Minor letter 2::A::1
20      Major letter ::G::60
20      Got '-' as minor value

25      Got 1
25      minor characters
25      Minor characters ::: A

30      Check quality for minor A

30      Got sequence , sequence
30      id AHRE9-5-D7
30      No of dashes before minor
30      character position      67
30      Quality value (
30      4) is less than 24 at position 4
30      Total No of minor characters quality is less than 24 is 1
30      Total No of minor characters
40      quality is greater than 24 is 0

45      AHRE9-5-D2  C-TCTGAGTTA;Accumulated SNP # : 0 S
45      AHRE9-5-H1  C-TCTGAGTTA;Accumulated SNP # : 0 S
45      AHRE9-5-C4  C-TTGAGTTA;Accumulated SNP # : 0 S
45      AHRE9-5-B5  C-TCTGAGTTA;Accumulated SNP # : 0 S
45      AHRE9-5-D5  C-TTGAGTTA;Accumulated SNP # : 0 S
45      AHRE9-5-A6  C-TCTGAGTTA;Accumulated SNP # : 0 S

```

## Substitute Specification for 09/964,059 - Unmarked "Clean" Copy

	AHRE9-5-B2	C-TCTGAGTTA;Accumulated SNP # : 0 S
	AHRE9-5-C3	C-TCTGAGTTA;Accumulated SNP # : 0 S
	AHRE9-5-C2	C-TCTGAGTTA;Accumulated SNP # : 0 S
	AHRE9-5-D3	C-TCTGAGTTA;Accumulated SNP # : 0 S
5	AHRE9-5-E2	C-TTGAGTTA;Accumulated SNP # : 0 S
	AHRE9-5-F2	C-TCTGAGTTA;Accumulated SNP # : 0 S
	AHRE9-5-E1	C-TCTGAGTTA;Accumulated SNP # : 0 S
	AHRE9-5-G2	C-TCTGAGTTA;Accumulated SNP # : 0 S
	AHRE9-5-G3	C-TCTGAGTTA;Accumulated SNP # : 0 S
10	AHRE9-5-H2	C-TTGAGTTA;Accumulated SNP # : 0 S
	AHRE9-5-D1	C-TTGAGTTA;Accumulated SNP # : 0 S
	AHRE9-5-F1	C-TTGAGTTA;ACcumulated SNP # : Q S
	AHRE9-5-D12	CATTCGAGTTA;Accumulated SNP # : 0 S
	AHRE9-5-B4	CAT-CGAGTTA;Accumulated SNP # : 0 S
15	AHRE9-5-D6	CAT-CGAGTTA;Accumulated SNP # : 0 S
	AHRE9-5-C1	CAT-CGAGTTA;Accumulated SNP # : 0 S
	AHRE9-5-A12	CAT-CGAGTTA;Accumulated SNP # : 0 S
	AHRE9-5-B11	CAT-AGAGTTA;Accumulated SNP # : 0 S
	AHRE9-5-D7	--AATAGAGTA;Accumulated SNP # : 1 S
20	AHRE9-5-H12	-----GGTTA;Accumulated SNP # : 0 S
	AHRE9-5-D4	C-TCTGAGTTA;Accumulated SNP # : 0 S
	AHRE9-5-C5	C-TCTGAGTTA;Accumulated SNP # : 0 S
	AHRE9-5-B1	C-TCTGAGTTA;Accumulated SNP # : 0 S
	AHRE9-5-B3	C-TCTGAGTTA;Accumulated SNP # : 0 S
25	AHRE9-5-A3	C-TCTGAGTTA;Accumulated SNP # : 0 S
	AHRE9-5-C6	CAT-CGAGTTA;Accumulated SNP # : 0 S
	AHRE9-5-F11	C-TCCGAGTTA;Accumulated SNP # : 0 S
	AHRE9-5-G11	C-TCCGAGTTA;Accumulated SNP # : 0 S
	AHRE9-5-C12	C-TTCGAGTTA;Accumulated SNP # : 0 S
30	AHRE9-5-E10	C-TCCGAGTTA;AcCumulated SNP # : 0 S
	AHRE9-5-C10	CTC-CGAGTTA;Accumulated SNP # : 0 S
	AHRE9-5-G12	CTCNCGAGTTA;Accumulated SNP # : 0 S
	AHRE9-5-D10	CATTCGAGTTA;Accumulated SNP # : 0 S
	AHRE9-5-D8	CATTCGAGTTA;Accumulated SNP # : 0 S
35	AHRE9-5-D9	CATCCGAGTTA;Accumulated SNP # : 0 S
	AHRE9-5-E11	C-TCCGAGTTA;Accumulated SNP # : 0 S
	AHRE9-5-C9	CAT-TGAGTTA;Accumulated SNP # : 0 S
	AHRE9-5-E8	TATTTCGAGTTA;Accumulated SNP # : 0 S
	AHRE9-5-B10	TCATTCGAGTTA;Accumulated SNP # : 0 S
40	AHRE9-5-D11	TCTTCGAGTTA;Accumulated SNP # : 0 S
	AHRE9-5-C8	CAT-CGAGTTA;Accumulated SNP # : 0 S
	AHRE9-5-B8	TCTTCGAGTTA;Accumulated SNP # : 0 S
	AHRE9-5-F8	TCTCNGAGTTA;Accumulated SNP # : 0 S
	AHRE9-5-H11	TCTCCGAGTTA;Accumulated SNP # : 0 S
45	AHRE9-5-A8	CAT-CGAGTTA;Accumulated SNP # : 0 S
	AHRE9-5-F12	C-TTCGAGTTA;Accumulated SNP # : 0 S
	AHRE9-5-E12	C-TCCGAGTTA;Accumulated SNP # : 0 S
	AHRE9-5-F7	CATCCGAGTTA;Accumulated SNP # : 0 S
	AHRE9-5-G10	C-TCCGAGTTA;Accumulated SNP # : 0 S
50	AHRE9-5-B9	C-TTCGAGTTA;Accumulated SNP # : 0 S
	AHRE9-5-C7	--CTTGAGT-A;Accumulated SNP # : 0 S
	AHRE9-5-F10	AATCCGAGTTA;Accumulated SNP # : 0 S
	AHRE9-5-C11	CATTCGAGTTA;Accumulated SNP # : 0 S
	AHRE9-5-A10	ACTCCGAGTTA;Accumulated SNP # : 0 S
55	AHRE9-5-F9	C-TCCGAGTTA;Accumulated SNP # : 0 S
	AHRE9-5-G8	C-TCCGAGTTA;Accumulated SNP # : 0 S

Left :

Right :

AGTTACAATGATATAATCTGGTCTTCCATTTTATAAAGCAGGCGTCATTAGACTGGACCCAAGTCCATCG

GTTGTTTTGTAAAGAAGCCGGA-

5 AAACTATCATGCCACTTCTCCANTCTTAATCACTAAAATAAAATTAAWA---

ATTAATTATCAAACCCCCAAATC-AATATAGTAAAGATTATTCTAAAA

Do you want to choose this into SNP data ?[y/n] n

\*\*\*\*\*

10 =====

Now consider the text window below which shows an alignment produced by

15 the software. Note the small numbers at the end of most of the lines (most are 0, some 1; one 17, one 22). When a discrepancy in the last two sequences having a quality score on the borderline is seen, and the number of "Accumulated SNPs" is high as it is shown in the last two lines, the discrepancy can be ignored as the large number indicates that 20 the sequence is of poor quality. This inference is good because real SNPs occur at a frequency of about 1 in 200 letters and the high numbers are much greater than one would expect. If it were not for these numbers, one would have to go and look at the sequence trace file to see if the discrepancy was real or not. Using this technique, it has never been observed that a discrepancy in a sequence with a large Accumulated SNP 25 number turns out to be a real SNP upon visual inspection of the trace data. Thus, time can be saved by avoiding to have to regularly view such trace data.

SEQ ID NOS: 221-239

=====

S13462.DPG-51-CP1 ACAATCCTTAA;Accumulated SNP # : 0 S  
 S13462.DPG-90-CP1 ACAATCCTTAA;Accumulated SNP # : 0 S  
 30 S13462.DPG-92-CP1 ACAATCCTTAA;Accumulated SNP # : 0 S  
 S13462.DPG-83-CP1 ACAATCCTTAA;Accumulated SNP # : 0 S  
 S13462.DPG-75-CP1 ACAATCCTTAA;Accumulated SNP # : 0 S  
 S13462.DPG-22-CP1 ACAATCCTTAA;Accumulated SNP # : 0 S  
 S13462.DPG-37-CP1 ACAATCCTTAA;Accumulated SNP # : 1 S  
 35 S13462.DPG-96-CP1 ACAATCCTTAA;Accumulated SNP # : 1 S

S13462.DPG-93-CP1 ACAATCCTTAA;Accumulated SNP # : 1 S  
 S13462.DPG-12-CP1 ACAATCCTTAA;Accumulated SNP # : 1 S  
 S13462.DPG-20-CP1 ACAATCCTTAA;Accumulated SNP # : 0 S  
 S13462.DPG-59-CP1 ACAATCCTTAA;Accumulated SNP # : 0 S  
 5 S13462.DPG-86-CP1 ACAATCCTTAA;Accumulated SNP # : 0 S  
 S13462.DPG-16-CP1 ACAATCCTTAA;Accumulated SNP # : 1 S  
 S13462.DPG-19-CP1 ACAATCCT--A-;Accumulated SNP # : 1 S  
 S13462.DPG-42-CP1 ACAAAACCT----;Accumulated SNP # : 17 S  
 S13462.DPG-14-CP1 ACAAACCTTAT;Accumulated SNP # : 22 S  
 10 Indicator ^  
 mar 204 404  
 Right Margin  
 Left :  
 CTCAGGTCCCACAGCAACAATATCATTCAAACGTGCAATTAAAACATAACACATAATATATAAGGTGAAGGT  
 15 ATTGAACATTACAGGATTATTAACACTGGCATTCCACTGTCTATTCTAAACATCAAGATGTGGGATGGAGCCTCGT  
 GCT  
 AGCTATAATGAAACACAATTAATATGAAATTAGTCCTGCCGATACAAT  
 Right : CTTAAAGGGCGAATTGTTAACCTGCAGGACTAG-----  
 ---  
 20 ---  
 Quality Values for Minor :::  
 18  
 Total No of minor charaters quality is less than 21 is 1  
 Total No of minor charaters quality is greater than 21 is 0  
 25 Do you want to choose this into SNP data ?[y/n]  
 =====

The inventive software has several useful features which distinguish it from other programs that use phred quality control data to find reliable discrepancies:

30 1) Other phred-based programs simply present the discrepancies that show a phred value above some arbitrary number. The problem is that it is quite common to find discrepancies with letters having quality values. Take the example below:

35 TAATTC  
 ATAATT  
 TAATTC  
 TAATTC

Note that the second sequence is "shifted" relative to the other three due to one single  
 40 sequencing mistake called an insertion, which is common. The alignment program is not perfect and does not always make the correct alignment by shifting the sequences

relative to one another. Even though the quality values for the letters A, T, A, A, T and T are very good, they are not SNPs but rather sequencing/alignment errors. Most other programs would output these letters as good candidate SNPs, so if the end-user did not go back to the data to inspect it valuable time and expense would be incurred by 5 designing genotyping experiments based on incorrect data.

The inventive program avoids this by visually presenting a local neighborhood of sequences to the end-user for those discrepancies that meet the phred threshold value. In other words, the program presents a block of sequences (such as the one above) so that an experienced user can recognize common errors such as this shift error.

10 Other common errors the end-user might notice are discrepancies in strings of sequence (such as GGGGG), or a phenomena called "bleedthrough". A conventional program relying just on phred score would select those mistakes and bad experiments would subsequently be designed. Since the inventive program shows the local sequence around this region for all the sequences, it is obvious to a trained molecular 15 biologist that the finding by the software is incorrect and should be discarded.

So one advantage of the software is that it presents a snapshot of the data, along with a query line asking if the user wishes to accept the data or not, so that invaluable human input is included in the SNP discovery analysis.

2) Another advantage is that the precise position and sequence that the 20 discrepancy occurs is readily apparent to the user. The example output above shows how this data is presented. Notice that each discrepancy is advantageously identified by using k = "column number". This is important in case the end-user wants to call up

the sequence data electropherogram, since it tells him which one to call up and where to go to see the relevant base. This is often done in different windows on the desktop. Visual inspection of raw DNA sequence data is the most reliable method of determining whether a discrepancy is valid. While the purpose of software is to eliminate such time consuming steps, in some cases borderline quality values require visual inspection. The presentation of the precise position and relevant file names for a discrepancy makes this step easy to perform.

3) Another advantage is that the end-user can specify a quality control value for a run of the program, then go back and repeat the run using a different quality control value. The quality for a position that meets the threshold requirements is also reported to the user so that borderline cases can be further reviewed.

4) Yet even another advantage is that the program presents the neighboring 200 letters of average sequence (for all of the individuals in an analysis) in front of and behind candidate SNP locations. This is important because when submitting SNP locations to a SNP consumables company (e.g., Orchid), one must submit the neighboring sequence as well so that the kit can be designed to assay this SNP in thousands of people.

5) Finally, another advantage is that the user can visualize deletion mutations, which do not have corresponding phred values. A unique attribute is afforded the software because of this functionality. The program can recognize reliable base deletion polymorphisms and present them to the user for visual inspection. In conventional programs, if a discrepancy is a deleted base there is no quality control

information to check since no data is produced for a non-base or deleted base (and there is consequently no phred value for the deleted base). This would eliminate the discovery of single base deletion polymorphisms. Deletion polymorphisms are common and, since the goal is to thoroughly document the various genetic haplotypes

5 in a population, a SNP finding program that can recognize deletion polymorphisms offers competitive advantages. Not knowing all of the variants in a gene sequence causes the resolution of haplotype-based studies to be sub-optimal, compared to being able to recognize all of the variants.

In an alternate embodiment, the software does not use actual DNA sequence

10 data files or chromatograms but rather accepts and utilizes sequence information in text format which is freely available and downloadable from publicly available databases. For quality control, an indirect measure of quality is used. For example, any discrepancy that occurs within a bleedthrough region, or within the neighborhood of discrepancy clusters is ignored.

15 It should be readily apparent and understood that the foregoing description is only illustrative of the invention and in particular provides preferred embodiments thereof. Various alternatives and modifications can be devised by those skilled in the art without departing from the true spirit and scope of the invention. E.g., gene data from human, animal, plant, or other may be utilized in connection with the methods.

20 Accordingly, the present invention is intended to embrace all such alternatives, modifications, and variations which fall within the scope of the appended claims.

What is claimed is:

CLAIMS

1. A method of processing gene sequence data with use of one or more computers, the method comprising:

5 reading, by the computer, gene sequence data corresponding to a gene sequence and coding sequence data corresponding to a plurality of coding sequences within the gene sequence;

10 identifying, by the computer following a set of primer selection rules, primer pair data within the gene sequence data, the primer pair data corresponding to a pair of primer sequences for one of the coding sequences, the set of primer selection rules including a first rule specifying that the primer pair data be obtained for a predetermined annealing temperature;

15 storing the primer pair data;

repeating the acts of identifying and storing such that primer pair data are obtained for each sequence of the plurality of coding sequences at the predetermined annealing temperature; and

20 simultaneously amplifying the plurality of coding sequences in gene sequences from three or more individuals at the predetermined annealing temperature using the identified pairs of primer sequences, such that a plurality of amplified coding sequences from the three or more individuals are obtained.

2. The method of claim 1, wherein the first rule further specifies that each primer sequence have a length that falls within one or more limited ranges of acceptable lengths.

5 3. The method of claim 1, wherein the set of primer selection rules includes a second rule specifying that a single primer pair be identified for two or more coding regions if they are sufficiently close together.

10 4. The method of claim 1, wherein gene family data associated with the gene sequence is read by the computer, and the set of primer selection rules includes a second rule specifying that the primer pair data be excluded from the gene family data.

15 5. The method of claim 1, further comprising:  
sequencing the plurality of amplified coding sequences to produce a plurality of nucleotide base identifier strings.

6. The method of claim 5, wherein the plurality of nucleotide base identifier strings includes nucleotide base identifiers represented by the letters G, A, T, and C.

20 7. The method of claim 6, further comprising:  
positionally aligning, by the computer, the plurality of nucleotide base identifier strings to produce a plurality of aligned nucleotide base identifier strings.

8. The method of claim 7, further comprising:

performing, by the computer, a comparison amongst aligned nucleotide base identifiers at each nucleotide base position of the plurality of aligned nucleotide base  
5 identifier strings.

9. The method of claim 8, performing the following additional acts at each nucleotide base position where a difference amongst aligned nucleotide base identifiers exists:

10 reading, by the computer, nucleotide base quality information associated with the aligned nucleotide base identifiers where the difference exists;

comparing, by the computer, the nucleotide base quality information with predetermined qualification data;

15 visually displaying, from the computer, the nucleotide base quality information for acceptance or rejection; and

if the nucleotide base quality information meets the predetermined qualification data and is accepted: providing and storing resulting data that identifies where the difference amongst the aligned base identifiers exists.

20 10. The method of claim 9, wherein the resulting data comprise single nucleotide polymorphism (SNP) identification data.

11. The method of claim 9, wherein the nucleotide base quality information comprise one or more phred values.

12. The method of claim 10, wherein after providing and storing all resulting  
5 data that identifies where the differences amongst the aligned nucleotide base identifiers exist, performing the following additional acts for each aligned nucleotide base identifier at each nucleotide base position where a difference exists:

comparing, by the computer, the nucleotide base identifier with a prestored nucleotide base identifier to identify whether the nucleotide base identifier is a variant;  
10 and

providing and storing, by the computer, additional resulting data that identifies whether the nucleotide base identifier is a variant.

13. The method of claim 12, wherein the additional resulting data comprises  
15 haplotype identification data.

14. The method of claim 13, wherein providing and storing additional resulting data comprises providing and storing a binary value of '0' for those nucleotide base identifiers that are identified as variants and a binary value of '1' for those  
20 nucleotide base identifiers that are not.

15. A computer program product comprising:

a computer-usable storage medium;

computer-readable program code embodied on said computer-usable storage

medium; and

5 the computer-readable program code for effecting the following acts on a computer:

reading gene sequence data corresponding to a gene sequence and coding sequence data corresponding to a plurality of coding sequences within the gene sequence;

10 identifying primer pair data within the gene sequence data by following a set of primer selection rules, the primer pair data corresponding to a pair of primer sequences for one of the coding sequences, the set of primer selection rules including a first rule specifying that the primer pair data be obtained for a predetermined annealing temperature;

15 storing the primer pair data;

repeating the acts of identifying and storing such that primer pair data are obtained for each sequence of the plurality of coding sequences at the predetermined annealing temperature, so that the plurality of coding sequences can be simultaneously amplified in gene sequences from three or more of individuals at the predetermined annealing temperature using the identified pairs of primer sequences to produce a plurality of amplified coding sequences from the three or more individuals.

16. The computer program product of claim 15, wherein the first rule further specifies that each primer sequence have a length that falls within one or more limited ranges of acceptable lengths.

5

17. The computer program product of claim 15, wherein the set of primer selection rules includes a second rule specifying that a single primer pair be identified for two or more coding regions if they are sufficiently close together.

10 18. The computer program product of claim 15, wherein gene family data associated with the gene sequence is read by the computer, and the set of primer selection rules includes a second rule specifying that the primer sequence data be excluded from the gene family data.

15 19. The computer program product of claim 15, wherein the plurality of amplified coding sequences are sequenced to produce a plurality of nucleotide base identifier strings.

20. The computer program product of claim 19, wherein the plurality of  
20 nucleotide base identifier strings includes nucleotide base identifiers represented by the letters G, A, T, and C.

21. The computer program product of claim 20, wherein the computer-readable program code is for effecting the following further acts on the computer:  
positionally aligning the plurality of nucleotide base identifier strings to produce  
a plurality of aligned nucleotide base identifier strings.

5

22. The computer program product of claim 21, wherein the computer-readable program code is for effecting the following further acts on the computer:  
performing a comparison amongst aligned nucleotide base identifiers at each  
nucleotide base position of the plurality of aligned nucleotide base identifier strings.

10

23. The computer program product of claim 22, wherein the computer-readable program code is for effecting the following additional acts at each nucleotide base position where a difference amongst aligned nucleotide base identifiers exists:

reading nucleotide base quality information associated with the aligned  
15 nucleotide base identifiers where the difference exists;

comparing the nucleotide base quality information with predetermined  
qualification data;

visually displaying the nucleotide base quality information for acceptance or  
rejection; and

20 if the nucleotide base quality information meets the predetermined qualification  
data and is accepted: providing and storing resulting data that identifies where the  
difference amongst the aligned base identifiers exists.

24. The computer program product of claim 23, wherein the resulting data comprise single nucleotide polymorphism (SNP) identification data.

5 25. The computer program product of claim 23, wherein the nucleotide base quality information comprise one or more phred values.

26. The computer program product of claim 24, wherein after providing and storing all resulting data that identifies where the differences amongst the aligned 10 nucleotide base identifiers exist, performing the following additional acts for each aligned nucleotide base identifier at each nucleotide base position where such difference exists:

comparing the nucleotide base identifier with a prestored nucleotide base identifier to identify whether the nucleotide base identifier is a variant; and

15 providing and storing additional resulting data that identifies whether the nucleotide base identifier is a variant.

27. The computer program product of claim 26, wherein the additional resulting data comprises haplotype identification data.

20

28. The computer program product of claim 27, wherein providing and storing additional resulting data comprises providing and storing a binary value of '0'

for those nucleotide base identifiers that are identified as variants and a binary value of '1' for those nucleotide base identifiers that are not.

29. A method of processing gene sequence data with use of one or more  
5 computers, the method comprising:

reading, by the computer, a plurality of nucleotide base identifier strings;

positionally aligning, by the computer, the plurality of nucleotide base identifier strings to produce a plurality of aligned nucleotide base identifier strings;

10 performing, by the computer, a comparison amongst aligned nucleotide base identifiers at each nucleotide base position of the plurality of aligned nucleotide base identifier strings;

15 performing, by the computer, a comparison amongst aligned nucleotide base identifiers at each nucleotide base position of the plurality of aligned nucleotide base identifier strings;

base identifiers exists:

15 at each nucleotide base position where a difference amongst aligned nucleotide

base identifiers exists:

reading, by the computer, nucleotide base quality information associated with the aligned nucleotide base identifiers where the difference exists;

20 comparing, by the computer, the nucleotide base quality information with predetermined qualification data;

visually displaying, from the computer, the nucleotide base quality information for acceptance or rejection; and

if the nucleotide base quality information meets the predetermined qualification data and is accepted: providing and storing resulting data that identifies where the difference amongst the aligned base identifiers exists.

5           30.     The method of claim 29, wherein the plurality of nucleotide base identifier strings includes nucleotide base identifiers represented by the letters G, A, T, and C.

31.     The method of claim 30, wherein the resulting data comprise single nucleotide polymorphism (SNP) identification data.

10

32.     The method of claim 31, wherein the nucleotide base quality information comprise one or more phred values.

33.     The method of claim 31, wherein after providing and storing all resulting data that identifies where the differences amongst the aligned nucleotide base identifiers exist, performing the following additional acts for each aligned nucleotide base identifier at each nucleotide base position where such difference exists:

comparing, by the computer, the nucleotide base identifier with a prestored nucleotide base identifier to identify whether the nucleotide base identifier is a variant;

20     and

providing and storing, by the computer, additional resulting data that identifies whether the nucleotide base identifier is a variant.

34. The method of claim 33, wherein the additional resulting data comprises haplotype identification data.

5        35. The method of claim 34, wherein providing and storing additional resulting data comprises providing and storing a binary value of '0' for those nucleotide base identifiers that are identified as variants and a binary value of '1' for those nucleotide base identifiers that are not.

10        36. A computer program product comprising:  
                a computer-usuable storage medium;  
                computer-readable program code embodied on said computer-usuable storage medium; and  
                the computer-readable program code for effecting the following acts on a  
15        computer:

                reading a plurality of nucleotide base identifier strings;  
                positionally aligning the plurality of nucleotide base identifier strings to produce a plurality of aligned nucleotide base identifier strings;  
                performing a comparison amongst aligned nucleotide base identifiers at each nucleotide base position of the plurality of aligned nucleotide base identifier strings;

performing a comparison amongst aligned nucleotide base identifiers at each nucleotide base position of the plurality of aligned nucleotide base identifier strings;

at each nucleotide base position where a difference amongst aligned nucleotide base identifiers exists:

reading nucleotide base quality information associated with the aligned nucleotide base identifiers where the difference exists;

comparing the nucleotide base quality information with predetermined qualification data;

visually displaying the nucleotide base quality information for acceptance or rejection; and

if the nucleotide base quality information meets the predetermined qualification data and is accepted: providing and storing resulting data that identifies where the difference amongst the aligned base identifiers exists.

37. The computer program product of claim 36, wherein the plurality of nucleotide base identifier strings includes nucleotide base identifiers represented by the letters G, A, T, and C.

38. The computer program product of claim 37, wherein the resulting data comprise single nucleotide polymorphism (SNP) identification data.

39. The computer program product of claim 38, wherein the nucleotide base quality information comprise one or more phred values.

5        40. The computer program product of claim 38, wherein after providing and storing resulting data that identifies where the differences amongst the aligned nucleotide base identifiers exist, performing the following additional acts for each aligned nucleotide base identifier at each nucleotide base position where such difference exists:

10           comparing the nucleotide base identifier with a prestored nucleotide base identifier to identify whether the nucleotide base identifier is a variant; and  
                 providing and storing additional resulting data that identifies whether the nucleotide base identifier is a variant.

15        41. The computer program product of claim 40, wherein the additional resulting data comprises haplotype identification data.

42. The computer program product of claim 41, wherein providing and storing additional resulting data comprises providing and storing a binary value of '0' 20 for those nucleotide base identifiers that are identified as variants and a binary value of '1' for those nucleotide base identifiers that are not.

43. A method of processing gene sequence data with use of one or more computers, the method comprising:

reading, by the computer, gene sequence data corresponding to a gene sequence and coding sequence data corresponding to a plurality of coding sequences within the

5 gene sequence;

identifying, by the computer following a set of primer selection rules, primer pair data within the gene sequence data, the primer pair data corresponding to a pair of primer sequences for one of the coding sequences, the set of primer selection rules including a first rule specifying that the primer pair data be obtained for a 10 predetermined annealing temperature and a second rule specifying that a single primer pair be identified for two or more coding regions if they are sufficiently close together;

storing, by the computer, the primer pair data; and

repeating the acts of identifying and storing such that primer pair data are obtained for the plurality of coding sequences at the predetermined annealing 15 temperature.

44. The method of claim 43, further comprising:

simultaneously amplifying the plurality of coding sequences in gene sequences from three or more of individuals at the predetermined annealing temperature using 20 the identified pairs of primer sequences, so that a plurality of amplified coding sequences from the three or more individuals are obtained.

45. The method of claim 43, wherein gene family data associated with the gene sequence is read by the computer, and the set of primer selection rules includes a third rule specifying that the primer sequence data be excluded from the gene family data.

# EFFICIENT METHODS AND APPARATUS FOR HIGH-THROUGHPUT PROCESSING OF GENE SEQUENCE DATA

5

## ABSTRACT OF THE DISCLOSURE

One disclosed method of processing gene sequence data includes the steps of reading gene sequence data corresponding to a gene sequence and coding sequence data corresponding to a plurality of coding sequences within the gene sequence; identifying and storing, by following a set of primer selection rules, primer pair data 10 within the gene sequence data for one of the coding sequences; repeating the acts of identifying and storing such that primer pair data are obtained for each sequence of the plurality of coding sequences; and simultaneously amplifying the plurality of coding sequences in gene sequences from three or more of individuals using the identified pairs of primer sequences. The set of primer selection rules include a rule specifying 15 that all of the primer pair data for the plurality of coding sequences be obtained for a predetermined annealing temperature, which allows for the subsequent simultaneous amplification of sequences from hundreds of individuals in a single amplification run.